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FACULTAD DE FARMACIA

DEPARTAMENTO DE NUTRICIÓN Y BROMATOLOGÍA,
TOXICOLOGÍA Y MEDICINA LEGAL

CARACTERIZACIÓN ESPECTROSCÓPICA Y AROMÁTICA DE VINAGRES ESPAÑOLES CON DENOMINACIÓN DE ORIGEN PROTEGIDA

Memoria presentada para
optar el Título de Doctor por la Universidad de Sevilla con la mención de
"Doctor Internacional" por la licenciada

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"Lo peor es temerle"

Antonio Cabello

A mis Padres, mi Hermana y Laura,

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ABREVIATURAS

°C	Grado centígrado
^{13}C-RMN	Resonancia magnética nuclear de carbono 13
^{1}H-RMN	Resonancia magnética nuclear de protones
3-AFC	Pruebas de elección forzada de tres alternativas
5-HMF	5-hydroxymethylfurfural
a.C.	Antes de cristo
AEDA	Aroma extract dilution analysis
ANOVA	Análisis de varianza
ASTM	Sociedad Americana para Pruebas y Materiales
ATR	Accesorio de reflectancia total atenuada
BOJA	Boletín Oficial Junta de Andalucía
C	Condado de Huelva
Cab	Intensidad de color por croma
CHARM	Combined hedonic aroma response measurement
CIS	Inyector con enfoque criogénico
cm	Centímetros
CR	Crianza
DHS	Extracción por espacio de cabeza dinámico
DOP	Denominación de origen protegida
DVB /Carboxen/PDMS	Divinilbenceno / Carboxen/ polidimetilsiloxano
EEM	Matriz de excitación-emisión
EFM	Espectroscopía de fluorescencia multidimensional
ELL	Extracción Líquido-Líquido
ELL	Extracción líquido-líquido
FD	Fusión de datos
FID	Detector iónico de llama
FTIR	Espectroscopia de transmisión de infrarrojo con transformada de Fourier
GC	Cromatografía de gases
GC-MS	Cromatografía de gases-espectrometría de masas
GC-MS-O	Cromatografía de gases-espectrometría de masas acoplado a olfatometría
GC-O	Cromatografía de gases acoplada con olfatometría
GR	Gran Reserva
HCM	Modelo de clasificación jerárquica
HPLC	Cromatografía líquida de alta resolución
HSE	Extracción por espacio de cabeza estático
HSQC	Espectros de coherencia cuántica única heteronuclear ^{1}H-^{13}C
HSSE	Extracción por sorción en espacio en cabeza estático
HS-SPME	Microextracción en fase sólida en espacio de cabeza
IGP	Indicación geográfica protegida
IR	Infrarrojo

IS	Estándar interno
ISO	*International Organization for Standardization*
J	Jerez
kg	kilogramo
L	Litro
LCN	*Local classifier per node*
LCPN	*Local classifier per parent node*
LVs	Variables latentes
MC	*Mean centering*
MCR	Resolución de curvas múltiples
MCR-ALS	Resolución Multivariante de Curvas-Mínimos Cuadrados Alternos
MF	Frecuencia modificada
mg	Miligramo
MHz	Megahercios
MIR	Espectroscopía de infrarrojo medio
mL	Mililitro
MM	Montilla-Moriles
mm	Milímetros
MS	Espectrometría de masas
MSD	Detector de espectros de masas
NIF	*Nasal Impact Frecuency*
NIR	Espectroscopía de infrarrojo cercano
NIR	Espectroscopía de infrarrojo cercano
nm	Nanómetros
N-PLS	Mínimos cuadrados parciales de múltiples vías
N-PLS-DA	Análisis discriminante N-PLS
OAV	Valor de actividad aromática
OENO	*Oenology*
OIV	Organización Internacional de la viña y el vino
OSH	Hiperplano de separación óptimo
PARAFAC	*Parallel factor Analysis*
PCA	Análisis de componentes principales
P-Comdim	*Predictive- Common Components and Specific Weights Analysis*
PCs	Componentes principales
PLS	Regresión de mínimos cuadrados parciales
PLS-DA	Análisis Discriminante sobre Mínimos Cuadrados Parciales
ppm	Partes por millón
PX	Pedro Ximénez
R^2	Coeficiente de determinación o regresión
RE	Reserva
s	Segundos
SBSE	*Stir Bar Sorptive Extraction*
SIMCA	Modelado suave independiente por analogía de clases
SMT	Smoothing
SO	Solera

SPE	Extracción en fase sólida
SVM	Máquina de vectores de soporte
SVN	Variable aleatoria normal tipificada
TDS2	Sistema de Desorción Térmica
TSG	Especialidad Tradicional Garantizada
UV-vis	Ultravioleta-visible
v/v	Volumen/volumen
WLS	*Weighted least squares*
λem	Longitud de onda de emisión
λex	Longitud de onda de excitación
µg	Microgramos
µL	Microlitros

RESUMEN

La autentificación de alimentos comprende el control de un amplio rango de parámetros fisicoquímicos y sensoriales, los cuales comúnmente han sido analizados por técnicas analíticas robustas, pero a su vez caras, laboriosas, y que requieren manipulación de la muestra y personal entrenado para su uso. Además, los compuestos que deben ser cuantificados para asegurar la autenticidad de un producto son numerosos y están continuamente cambiando debido a la mayor sofisticación de los métodos de adulteración. En consecuencia, hay una creciente necesidad de buscar métodos analíticos rápidos, sencillos, baratos, robustos, efectivos y que no requieran apenas manipulación de la muestra, capaces de autentificar alimentos, clasificarlos y detectar adulteraciones o fraudes. Con esta finalidad, las técnicas espectroscópicas, combinadas con técnicas quimiométricas, han demostrado ser herramientas útiles para la caracterización y autentificación de alimentos, así como para la detección de posibles adulteraciones o fraudes.

Hoy en día, uno de los productos alimentarios que se están viendo afectados por fraudes y falsificaciones, y que por tanto requieren de su autentificación, son aquellos que se encuentran protegidos bajo una Denominación de Origen Protegida (DOP). Y entre los productos amparados bajo una DOP encontramos al vinagre de vino. En España son tres las DOP de vinagre de vino reconocidas: “Vinagre de Jerez”, “Vinagre del Condado de Huelva”, y “Vinagre de Montilla-Moriles”, siendo las tres producidas en el sur de España, Andalucía. Estos vinagres de vino con DOP cuentan con una complejidad química, aromática y sensorial, originada e influenciada por el material de partida, el método de producción, los compuestos formados durante la fermentación, y en algunos casos, los producidos durante el tiempo en el que se envejecen en barricas de madera. Por tanto, uno de los parámetros principales necesarios para determinar la calidad de estos vinagres y diferenciarlos de otros y entre sí, es el estudio de su perfil volátil, aromático y sensorial, el cual no se había realizado hasta la fecha con los vinagres de vino de las tres DOP españolas.

En este contexto, la presente Tesis Doctoral titulada “Caracterización espectroscópica y aromática de vinagres españoles con denominación de origen protegida” tiene como uno de sus objetivos principales, ofrecer una nueva metodología analítica que permita la caracterización y autentificación de los vinagres de vino españoles con DOP, productos de alto valor en la dieta Mediterránea, para garantizar que el consumidor reciba un producto con total garantía en cuanto a seguridad, origen, métodos de producción, DOP y categoría, utilizando para ello un método de control económico, rápido y sencillo que sirva como alternativa a los métodos tradicionales. Para la consecución de este objetivo se propone emplear técnicas

espectroscópicas en combinación con técnicas quimiométricas, debido a que son técnicas no destructivas, sensibles, rápidas y de relativo bajo coste que han demostrado su competitividad en el campo de la caracterización y clasificación de alimentos. Así, en la presente Tesis Doctoral se analizaron los vinagres de vino españoles con DOP mediante espectroscopía de infrarrojo medio y cercano (ATR-FTIR y NIR), espectroscopía de fluorescencia multidimensional (EFM), espectroscopía de ultravioleta-visible (UV-Vis) y resonancia magnética nuclear de protones (^{1}H-RMN). Además, se les realizó el análisis de isótopos estables de carbono y oxígeno con el fin de asegurar su autenticidad y detectar posibles fraudes. Por otro lado, el proyecto de Tesis Doctoral también tiene como objetivo realizar una caracterización aromática de estos vinagres de vino con DOP mediante la determinación de sus perfiles volátiles, aromáticos y sensoriales por cromatografía de gases-espectrometría de masas (GC-MS), análisis olfatométricos (GC-MS-O) y análisis sensorial, respectivamente, así como la determinación de los compuestos volátiles y aromáticos que puedan servir como marcadores de calidad y autenticidad de cada DOP. El proyecto de Tesis Doctoral culmina con el desarrollo de una herramienta informática basada en el mejor modelo de clasificación obtenido en combinación con métodos de análisis multivariante, que sea capaz de conseguir los objetivos citados. Los objetivos de la presente Tesis Doctoral se enmarcan en un objetivo general de la industria vinagrera centrada en la obtención de procedimientos de trazabilidad integral, aumentando de esa forma la confianza del consumidor y reforzando la competitividad de las empresas en un mercado agroalimentario cada vez más competitivo.

Con respecto a la caracterización y autentificación espectroscópica, caben destacar ciertos resultados obtenidos por las distintas técnicas espectroscópicas estudiadas. Así, el análisis de los vinagres de vino por infrarrojo medio (ATR-FTIR) y cercano (NIR) permitieron una diferenciación de las categorías dentro de cada DOP, aunque no lograron obtenerse buenos modelos de clasificación (clasificación correcta para algunas categorías de sólo el 58%). Por otro lado, la espectroscopía de fluorescencia multidimensional permitió una buena clasificación de las DOP y sus categorías (alrededor de un 90% de clasificación correcta), así como la detección y cuantificación de la adición de caramelo de mosto a estos vinagres, siendo éste estudiado por primera vez. Además, el análisis por ^{1}H-RMN permitió la identificación de ciertos compuestos responsables de la diferencia entre DOPs independientemente de la categoría a la que pertenecían los vinagres de vino. Con el fin de buscar el mejor modelo de clasificación, se realizó también una fusión de datos espectroscópicos, cuyos resultados mejoraron los modelos de clasificación obtenidos de manera individual por cada una de las técnicas anteriores. Por otro lado, el análisis de isotopos estables del carbono (δ^{13}C) y oxígeno (δ^{18}O) demostró que las

muestras analizadas no estaban adulteradas, así como permitió la diferenciación de los mismos según el origen geográfico español, diferenciando vinagres de vino del norte de España de los del sur, e incluso diferenciando las tres DOPs entre sí.

Sin embargo, dentro de todos los modelos de clasificación desarrollados por las diferentes técnicas espectroscópicas estudiadas, caben destacar por encima de los demás, los obtenidos por la espectroscopía de UV-vis, mediante un equipo portátil. Esta técnica es una de las más sencillas, rápidas y económicas de entre las diferentes técnicas espectroscópicas, y proporcionó los mejores modelos de clasificación, e incluso permitió el desarrollo de modelos jerárquicos de clasificación que eran capaces de diferenciar: el método de producción de un vinagre de vino, tradicional o rápido, o lo que es lo mismo, vinagres con DOP de vinagres sin DOP; el tiempo de envejecimiento o categorías; vinagres de vino de diferentes DOP; así como las diferentes categorías dentro de cada DOP. Además, el ser un equipo portátil, nos acerca lo máximo posible a la implementación en un futuro en bodegas u organismos de control. Debido a los excelentes resultados obtenidos por esta técnica, junto con todas las ventajas citadas anteriormente, los resultados se usaron para construir la herramienta informática clasificatoria de vinagres llamada “VinegarScan”, consiguiendo con esto cumplir con el último objetivo de esta Tesis Doctoral.

Por otro lado, con respecto a la caracterización aromática de los vinagres de vino españoles con DOP, primero se realizó un estudio comparativo de las técnicas de extracción más empleadas para este tipo de matriz, el cual permitió seleccionar la técnica de extracción por sorción en espacio en cabeza estático (HSSE) como la técnica de extracción más adecuada para el análisis de estos vinagres de vino con DOP, debido a que extraía un mayor número de compuestos, permitía una mejor diferenciación de las DOPs y categorías y contaba con más ventajas que las otras técnicas estudiadas. Una vez seleccionada la técnica de extracción, todas las muestras fueron analizadas por HSSE-GC-MS, consiguiéndose una diferenciación y clasificación de las tres DOP y sus categorías según el perfil volátil, así como la selección de una serie de marcadores volátiles responsables de esta diferenciación. Además, se llevó también a cabo el estudio del aroma de estos vinagres mediante GC-MS-O y análisis sensorial el cual permitió una diferenciación de las DOPs, obteniéndose una serie de aromas de impacto y atributos sensoriales claves para su diferenciación. Así, en rasgos generales, los vinagres de la DOP Vinagre de Jerez mostraron un mayor porcentaje de odorantes de impacto responsables de aromas verdes y herbáceos, y de notas dulces, especiadas y licorosas para su categoría Pedro Ximénez; Vinagre de Montilla-Moriles mostró un mayor porcentaje de odorantes de impacto responsables de aromas a mantequilla-láctico-queso, con de aromas dulces, tostadas y

especiadas para la categoría Pedro Ximénez; y Vinagre de Condado de Huelva mostró un mayor porcentaje de odorantes de impacto responsables de aromas químicos, punzantes y afrutados.

Los resultados obtenidos son sin duda de gran interés ya que se ha demostrado que los vinagres de vino españoles con DOP muestran unas características químicas y organolépticas que le otorgan una calidad diferencial con respecto a otros vinagres, y que hacen posible su autentificación y clasificación por diferentes técnicas analíticas. Además, se ha demostrado que es posible diferenciar incluso los vinagres de entre las tres DOPs y de sus categorías, a pesar de ser producidos de manera muy similar mediante métodos tradicionales, y en regiones geográficas muy próximas, lo que dificulta su diferenciación, pero a su vez les da mayor valor a los resultados obtenidos en esta Tesis Doctoral. Asimismo, el conocimiento generado a través de la caracterización realizada de cada una de las muestras por numerosas técnicas analíticas, tanto a nivel espectral como a nivel aromático, permitirá dar un valor añadido a la producción y comercialización de estos vinagres españoles con DOP. Por otro lado, se ha demostrado la capacidad de autentificación y clasificación de los vinagres de vino españoles con DOP mediante técnicas espectroscópicas en combinación con técnicas quimiométricas, con la ventaja de que son técnicas rápidas, económicas y que no requieren apenas de manipulación de muestra, lo cual supone una ventaja importante frente a las técnicas analíticas que se utilizaban tradicionalmente como métodos de control.

Esta Tesis Doctoral ha dado lugar a 10 trabajos de investigación, 9 de ellos publicados en revistas indexadas con alto índice de impacto, y 3 de ellos galardonados con el “Premio a la publicación científica del mes de la Facultad de Farmacia”. Además, los resultados han dado lugar a 15 comunicaciones a congresos internacionales y a 3 capítulos de libro. Asimismo, la presente Tesis Doctoral ha generado un software informático, que ha sido inscrito en el Registro de Propiedad Intelectual de la US, el cual ha causado gran interés en los Consejos Reguladores y sistemas de auditorías asociados, así como a bodegueros, que podrán poner estos avances en práctica como métodos de control de sus vinagres. Además, los conocimientos generados en esta Tesis Doctoral podrán ser aplicados a otros alimentos españoles con DOP como vinos, aceites, quesos o jamón, con objeto de obtener modelos de clasificación robustos, capaces incluso de detectar muestras adulteradas.

SUMMARY

Food authentication involves the control of a wide range of physicochemical and sensory parameters, which have been commonly analysed by robust analytical techniques, which are also expensive and laborious, and require sample handling and trained personnel for their use. In addition, the compounds that might be quantified to ensure the authenticity of a product are numerous and are continuously changing due to the greater sophistication of the adulteration methods. Consequently, there is a growing need to look for rapid, simple, cheap, robust and effective analytical methods that do not require sample handling, capable of authenticating food, classifying them and detecting adulterations or frauds. For this purpose, spectroscopic techniques, combined with chemometric techniques, have proven to be useful tools for the characterization and authentication of food products, as well as for the detection of possible adulterations or frauds.

Nowadays, one of the food products that are being affected by frauds and counterfeiting, which therefore require their authentication, are those that are protected under a Protected Designation of Origin (PDO). Among the products covered under a PDO, the wine vinegar is found. There are three recognized wine vinegar PDOs in Spain: "Vinagre de Jerez", "Vinagre del Condado de Huelva", and "Vinagre de Montilla-Moriles", all three being produced in southern Spain, in Andalusia. These PDO wine vinegars have a chemical, aromatic and sensorial complexity, originated and influenced by the raw material, the production method, the compounds formed during the fermentation, and in some cases, those produced during the period of time in which they are aged in wooden barrels. Therefore, one of the main parameters needed for determining these vinegars' quality and for differentiating them from others and between each other, is the study of their volatile, aromatic and sensory profiles, which had not been done to date with the three Spanish PDO wine vinegars.

In this context, the present Doctoral Thesis entitled "Spectroscopic and aromatic characterization of Spanish vinegars with protected designation of origin" has, as one of its main objectives, to offer a new analytical methodology that allows the characterization and authentication of the Spanish wine vinegars with PDO, products highly recognized in the Mediterranean diet, to ensure that the consumer receives a product with full security in terms of safety, origin, production methods, PDO and category, using an economic, fast and simple control method to serve as an alternative to traditional methods. To achieve this objective, spectroscopic techniques are proposed to be used, in combination with chemometric techniques, because they are non-destructive, sensitive, rapid and relatively low cost techniques

that have demonstrated their competitiveness in the field of food characterization and classification. Thus, in this Doctoral Thesis, Spanish PDO wine vinegars were analysed using medium and near infrared spectroscopy (ATR-FTIR and NIR), multidimensional fluorescence spectroscopy (EFM), ultraviolet-visible spectroscopy (UV-Vis) and proton nuclear magnetic resonance (^{1}H-NMR). In addition, they were analysed by stable carbon and oxygen isotopes in order to ensure their authenticity and to detect frauds. On the other hand, the Doctoral Thesis project also aims to perform an aromatic characterization of these PDO wine vinegars by determining their volatile, aromatic and sensorial profiles by gas chromatography-mass spectrometry (GC-MS), olfactometric (GC-MS-O) and sensory analysis, respectively, as well as the determination of volatile and aromatic compounds that could be considered markers of quality and authenticity of each PDO. The Doctoral Thesis project culminates with the development of a software based on the best classification model obtained in combination with multivariate analysis methods, which can be able to achieve the aforementioned objectives. The aims of this Doctoral Thesis are framed within a general objective of the vinegar industry that is focused on obtaining comprehensive traceability procedures, increasing consumer confidence and strengthening the competitiveness of companies in an increasingly competitive agri-food industry.

Regarding the characterization and spectroscopic authentication, it is worth mentioning certain results obtained by the different spectroscopic techniques studied. Thus, the analysis of the PDO wine vinegars by medium (ATR-FTIR) and near (NIR) infrared allowed a differentiation of the categories within each PDO, although good classification models could not be obtained (correct classification for some categories around 58%). On the other hand, the multidimensional fluorescence spectroscopy (EFM) allowed a good PDO and category classification (around 90% of correct classification), as well as it allowed the detection and quantification of the addition of grape-must caramel to these vinegars, being studied for the first time. Furthermore, the analysis of these PDO wine vinegars by ^{1}H-NMR allowed the identification of certain compounds responsible for the difference between PDOs regardless of the category to which the wine vinegars belonged. In order to find the best classification model, a fusion of spectroscopic data was also performed. The data fusion models obtained improved the classification, providing a more efficient differentiation, than the models based on single methods. On the other hand, stable isotopes analysis of carbon (δ^{13}C) and oxygen (δ^{18}O) showed that the analysed samples were not adulterated, as well as allowed the differentiation of the wine vinegars according to the Spanish geographical origin, differentiating wine vinegars from

the north from those of the south of Spain, and even differentiating the three PDOs from each other.

However, within all the classification models developed by the different spectroscopic techniques studied up to date, those obtained by UV-vis spectroscopy, by means of a portable equipment, are worth highlighted. This technique is one of the simplest, fastest and most economical spectroscopic technique studied in this project, and moreover, it provided the best classification models, and even allowed the development of a hierarchical classification model that was able to differentiate: the method of production of a wine vinegar, traditional or fast, or what is the same, vinegars with PDO from vinegars without PDO; the aging time or the categories; different PDOs of wine vinegars; as well as the different categories within each PDO. In addition, as it was a portable equipment, it brings a high possibility to be implemented in wineries or control agencies in the future. Due to the excellent results obtained by this technique, together with all the advantages above mentioned, the results were used to build a software tool called "VinegarScan", achieving with it the last objective of this Doctoral Thesis.

On the other hand, with respect to the aromatic characterization of the Spanish PDO wine vinegars, firstly, a comparative study of the most used extraction techniques for this type of food matrix was carried out, which allowed to select the extraction technique headspace sorptive extraction (HSSE) as the most appropriate extraction technique for the analysis of these PDO wine vinegars, because it extracted a greater number of compounds, allowed a better differentiation of the PDOs and categories and had more advantages than the other techniques studied. Once the extraction technique was selected, all the samples were analysed by HSSE-GC-MS, obtaining a good differentiation and classification of the three PDOs and their categories according to the volatile profile, as well as allowing the selection of some volatile markers responsible for this differentiation. Furthermore, the study of the aroma of these vinegars was also carried out by GC-MS-O and sensory analysis which also allowed a differentiation of the PDOs, allowing the selection of some impact aromas and key sensory attributes responsible for the differentiation. Thus, in general terms, wine vinegars from the Vinagre de Jerez PDO showed a higher percentage of green and herbaceous impact odorants, with sweet, spicy and liqueur notes for their Pedro Ximénez category; Vinagre de Montilla-Moriles PDO showed a higher percentage of impact odorants responsible for a butter-lactic-cheese aroma, with sweet, toasted and spicy nuances for the Pedro Ximénez category; and Vinagre de Condado de Huelva showed a higher percentage of chemical, pungent and fruity impact odorants.

The results obtained are undoubtedly of great interest since it has been demonstrated that Spanish PDO wine vinegars show some chemical and organoleptic characteristics that provide them a differential quality with respect to other vinegars, and that make possible their authentication and classification by different analytical techniques. In addition, it has been shown that it is possible to differentiate even the vinegars from the three PDOs and their categories, despite being produced by traditional methods in a very similar way, and in very close geographical regions, which makes their differentiation more difficult, but in turn, it gives greater value to the results obtained in this Doctoral Thesis. In addition, the knowledge generated through the characterization of each of the samples by numerous analytical techniques, both at spectrally and aromatic levels, will give added value to the production and marketing of these Spanish PDO wine vinegars. Likewise, the ability to authenticate and classify Spanish PDO wine vinegars has been demonstrated by means of spectroscopic techniques in combination with chemometrics, with the advantage that they are fast, economical techniques that do not require just sample handling, which it is an important advantage over the analytical techniques that were traditionally used as control methods.

This Doctoral Thesis has given rise to 10 research works, 9 of them published in indexed journals with high impact index, and 3 of them awarded with the "Scientific Publication Prize of the month of the Faculty of Pharmacy". In addition, the results have led to 15 communications to international conferences and 3 book chapters. In addition, this Doctoral Thesis has generated a computer software, which has been registered in the Intellectual Property Registry of the US, which has caused great interest in the Regulatory Boards and associated audit systems, as well as winemakers, who may put these advances in practice as control methods of their own vinegars. In addition, the knowledge generated in this Doctoral Thesis can be applied to other Spanish food products with PDO such as wines, oils, cheeses or ham, in order to obtain robust classification models, being even able to detect adulterated samples.

1. INTRODUCCIÓN

INTRODUCTION

1.1. EL VINAGRE

El vinagre se define, según la última reglamentación técnico-sanitaria de 2012 (BOE-A-2012-5529, 2012), como "*líquido apto para el consumo humano resultante de la doble fermentación alcohólica y acética de productos de origen agrario*". Por lo tanto, el vinagre es, de forma resumida, un líquido apto para el consumo humano, que contiene una cantidad específica de ácido acético y agua.

El vinagre es uno de los productos fermentados más antiguos del mundo, remontándose su historia a alrededor del año 2000 a.C., el cual ha sido considerado durante mucho tiempo como un subproducto de menor valor entre los productos alimentarios fermentados (Solieri & Giudici, 2009). A pesar de no tener valor nutricional, su carácter ácido debido al alto contenido en ácido acético (hasta la descripción del ácido sulfúrico, fue el ácido más fuerte conocido), facilitó su uso como conservante de alimentos gracias a su actividad antimicrobiana (Frias, Martinez-Villaluenga, & Peñas, 2017; Murooka & Yamshita, 2008). En la actualidad, el vinagre es un producto imprescindible en los hogares de todo el mundo, siendo ampliamente utilizado como conservante, agente saborizante y en algunos países, incluso como una bebida saludable. Esto hace que su demanda esté en crecimiento. Además, el crecimiento de las poblaciones, el aumento de los ingresos disponibles, el aumento de la conciencia de salud entre los consumidores y la industria de alimentos y bebidas son los principales factores que están impulsando el mercado del vinagre. El interés por cocinar comidas gourmet y étnicas ha aumentado entre muchos consumidores, lo que ha llevado a la venta de varios aderezos, la mayoría de los cuales utilizan vinagre como uno de los ingredientes clave. Además, aunque el vinagre se consume principalmente en la industria de alimentos y bebidas, también encuentra aplicaciones en la industria de la salud y la limpieza.

En general, el consumo de vinagre, al igual que ocurre con otras bebidas fermentadas, está asociado a un determinado patrón dietético con intensas connotaciones culturales y geográficas. Así, en los países mediterráneos, la mayoría del vinagre se usa directamente o se agrega a las ensaladas o a vegetales crudos o cocidos, siendo directa la apreciación de las características organolépticas del vinagre, y, por tanto, los vinagres de "calidad" están estrechamente relacionados con estos patrones de consumo. Por el contrario, en otros países, la mayoría del vinagre se utiliza para marinar, escabechar o como parte de salsas, y el impacto organoléptico, y por tanto sus cualidades, son menos evidentes. Estas diferencias en consumo también vienen acompañadas de diferencias en la producción según la región o el país, dando lugar a diferentes tipos de vinagres (Garcia-Parrilla, Torija, Mas, Cerezo, & Troncoso, 2016).

1.1.1. TIPOS DE VINAGRE

El vinagre se obtiene mediante dos procesos consecutivos de fermentación, alcohólica y acética en el que se realiza la conversión de diferentes fuentes de carbohidratos (azúcares fermentables) que pueden provenir de distintas materias primas agrícolas (Mas, Torija, García-Parrilla, & Troncoso, 2014b; Tesfaye, Morales, García-Parrilla, & Troncoso, 2002). Durante la fermentación alcohólica, las levaduras son los primeros microorganismos que participan, siendo las encargadas de convertir el azúcar en alcohol. Tras ellas, las bacterias acéticas son las que convierten este etanol en ácido acético, conociéndose a este último proceso como "acetificación" debido a su estricto requerimiento de oxígeno (Mas, Torija, García-Parrilla, & Troncoso, 2014a). Además, la elaboración de los vinagres presentes en el mercado puede realizarse principalmente por dos tipos de acetificación: acetificación por cultivo sumergido o por cultivo superficial. Teniendo en cuenta la variabilidad en la producción, los vinagres pueden ser clasificados en función del tipo de sustrato o materia prima empleada o del método usado para su elaboración.

1.1.1.1. Clasificación según la materia prima

De acuerdo a esta primera clasificación, en la **Tabla 1** se muestran las materias primas comúnmente utilizadas para obtener vinagre. Estas materias primas *s*on principalmente materiales vegetales como frutas (uva, manzana, mango, dátiles, etc.) y cereales (arroz, malta), con algunas excepciones como el suero de leche y la miel, abarcando una amplia variedad de orígenes.

Además, se pueden encontrar en el mercado otros vinagres, como el vinagre blanco destilado, siendo un vinagre obtenido directamente a partir de alcohol diluido (originado de diversas fuentes como caña de azúcar, granos de maíz, etc.), el cual no tiene ningún periodo de maduración. Este vinagre, de baja calidad, es muy comercializado ya que en la cultura de muchos países, el vinagre sigue siendo un producto poco valorado, lo que hace que no se tenga en cuenta su calidad (Solieri & Giudici, 2009). Además, a partir de estos vinagres se pueden obtener los llamados vinagres aromatizados o vinagres de especias, los cuales se producen mediante maceración o agregación de frutas, flores o especias aportándoles un sabor y aroma característicos y que han sido añadidos en 2012 dentro de la categoría de “vinagres” (BOE-A-2012-5529, 2012; Frias et al., 2017).

Tabla 1. Clasificación de los vinagres según sustrato, nombre y región o país de producción y distribución.

SUSTRATO	NOMBRE	REGIÓN/PAÍS (PRODUCCIÓN & DISTRIBUCIÓN)
Uva	Vinagre de vino	Global
	Vinagre balsámico	Global
	Vinagre de vino rojo	Global
	Vinagre de vino blanco	Global
	Vinagre de Jerez	Global
	Vinagre de Condado de Huelva	Global
	Vinagre de Montilla-Moriles	Global
	Vinagre Balsámico Tradicional de Módena	Global
Manzana	Vinagre de sidra	US, Canadá
Frutas (mango, kaki, bayas)	Vinagre de frutas	Este y sudeste de Asia
Dátiles	Vinagre de dátiles	Medio Oriente
Coco	Vinagre de coco	África tropical
Arroz	Vinagre de arroz	China, Japón, Corea
	Korosu	China, Japón, Corea
Malta	Vinagre de malta	EEUU, Europa del Norte
	Vinagre de malta destilado	EEUU, Europa del Norte
Suero (subproductos lácteos)	Vinagre de suero	Europa
Miel	Vinagre de miel	Global

Nota: Tabla adaptada del Capítulo de libro: Vinegar, FOODINTEGRITY HANDBOOK. A guide to food authenticity issues and analytical solutions, Editors Jean-François Morin & Michèle Lees, Eurofins Analytics France, 2018. https://doi.org/10.32741/fihb. (Callejón, Ríos-Reina, Morales, & Troncoso, 2018). (**ANEXO I**).

Respecto a la producción, a nivel mundial el vinagre blanco destilado es el más producido y comúnmente utilizado en el hogar, la industria alimentaria y farmacéutica, debido a su bajo coste. Sin embargo, hay ciertos países cuya producción de vinagres distintos al vinagre blanco destilado es mayor. Así, el vinagre de arroz es el tipo más común en Asia, aunque también se encuentran otros tipos, muchos de ellos siguiendo sistemas de producción tradicionales. En otros países, sobretodo del norte de Europa, los vinagres de frutas, especialmente los de manzana, están obteniendo una gran aceptación por parte del consumidor debido a que se le atribuyen beneficios para la salud por a su riqueza en aminoácidos, vitaminas y sustancias minerales entre otros (Liu, He, & Wang, 2008).

Por otro lado, el vinagre de vino es el más producido y consumido en los países mediterráneos, especialmente España, Francia e Italia, expandiéndose considerablemente en los últimos años a otros muchos países de todo el mundo debido a las nuevas tendencias gastronómicas (Callejón et al., 2018). Estos vinagres están teniendo una gran importancia en el mercado internacional. Geográficamente, Europa representa en la actualidad el mayor mercado para el vinagre de vino, seguida por América del Norte y la región del Pacífico de Asia. De hecho, en 2016, el vinagre balsámico exhibió un claro dominio respecto a su participación en el mercado (Callejón et al., 2018). Así, el vinagre balsámico de Módena es uno de los más reconocidos a nivel mundial, llegando a venderse en pequeñas cantidades (100 mL) a un altísimo precio, principalmente debido al gran número de años de envejecimiento a los que están sometidos (incluso más de 40 años).

1.1.1.2. Clasificación según el método de producción

Los vinagres también pueden diferenciarse en dos tipos según su sistema de producción: cultivo sumergido o cultivo superficial. En el primer tipo de cultivo, el cultivo sumergido, las bacterias acéticas se encuentran sumergidas libremente en el seno del líquido a fermentar, por el que se hacen circular burbujas de aire (sólo o enriquecido con oxígeno) a través de la biomasa, favoreciéndose la fermentación y, por tanto, consiguiendo acetificaciones muy rápidas. Aunque los primeros recipientes para el procesamiento de cultivos sumergidos se hicieron de madera, los recipientes habituales son el acero inoxidable, que es más higiénico y resistente a las condiciones ácidas. Un modelo de este procedimiento es el *Acetificador de Frings,* representado en la Figura 1, y que constituye la base de la biotecnología vinagrera actual (Hromatka & Ebner, 1959). Este método de producción por cultivo sumergido es muy utilizado debido a que se consiguen grandes cantidades de vinagre en cortos periodos de tiempo, necesitando menos espacio e incluso teniendo la posibilidad de automatizar el proceso. Así se obtienen de forma rápida los vinagres comerciales actuales de menor precio y calidad. Esta menor calidad es debido, entre otras cosas, a que el elevado suministro de aire puede causar sobreoxidación del producto y arrastre de componentes volátiles. Las limitaciones se pueden compensar con el envejecimiento posterior en barriles o sumergiendo fragmentos o astillas de madera, lo que puede contribuir a la obtención de nuevos caracteres organolépticos que pueden aumentar la calidad del producto final.

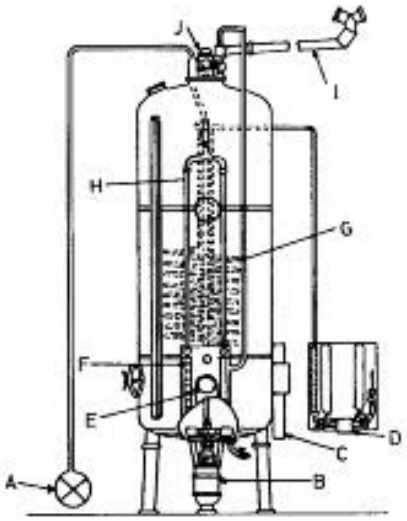


Figura 1. Acetificador de Frings. A, bomba de carga; B, aireador y motor; C, dispositivo para determinación de alcohol residual; D, válvula entrada agua refrigeración; E, termostato regulador; F, rotámetro; G, serpentín de refrigeración; H, entrada de aire; I, salida de gases; J, dispositivo antiespuma. Fuente: (Polo, Sanchez-Luengo, 1991).

El segundo método de producción, “cultivo superficial”, es el que habitualmente se aplica en los vinagres tradicionales. En este procedimiento la transformación del etanol en ácido acético se realiza mediante un cultivo estático de bacterias acéticas en la interfase entre el líquido y el aire. Los barriles se llenan hasta 2/3 de su capacidad para dejar una cámara de aire, que se mantiene en contacto con el aire exterior utilizando una o varias aberturas. Las bacterias acéticas se encuentran en contacto directo con el oxígeno gaseoso, situadas bien en la interfase líquido/gas o bien fijadas a soportes de materiales tales como virutas. Uno de los métodos más antiguos para fabricar vinagre dentro de los cultivos superficiales es el método Orleans (Figura 2). Este método incluye orificios laterales en los toneles para la circulación de aire y agrega un tubo de vidrio recto, que llega hasta casi el fondo del barril, para permitir que el sustrato sea renovado evitando la alteración de la "madre del vinagre" o velo bacteriano de la superficie. Esta madre es una película formada por los microorganismos transformadores, es decir, de bacterias acéticas, que se desarrollan en la superficie debido a la necesidad de oxígeno. Los vinagres producidos por este sistema tradicional generalmente se consideran de alta calidad debido a su complejidad organoléptica, ya que tienen el aroma y sabor propio de la lentitud de la acetificación, que se debe principalmente al metabolismo de las bacterias acéticas y al proceso de envejecimiento que se inicia en la propia etapa de producción. Sin embargo, este proceso es muy lento, y la producción de vinagre puede llevar de meses a años. Para aumentar la velocidad de acetificación, y por tanto disminuir el tiempo de producción, se han desarrollado otros métodos, como el método Luxemburgués, que a diferencia del de Orleans, emplea unas virutas

de haya aumentado la superficie de acetificación y por tanto su velocidad. Otras desventajas que presenta este tipo de cultivo son los posibles aumentos de temperatura difíciles de controlar, las pérdidas de alcohol por evaporación y las mayores necesidades de espacio.

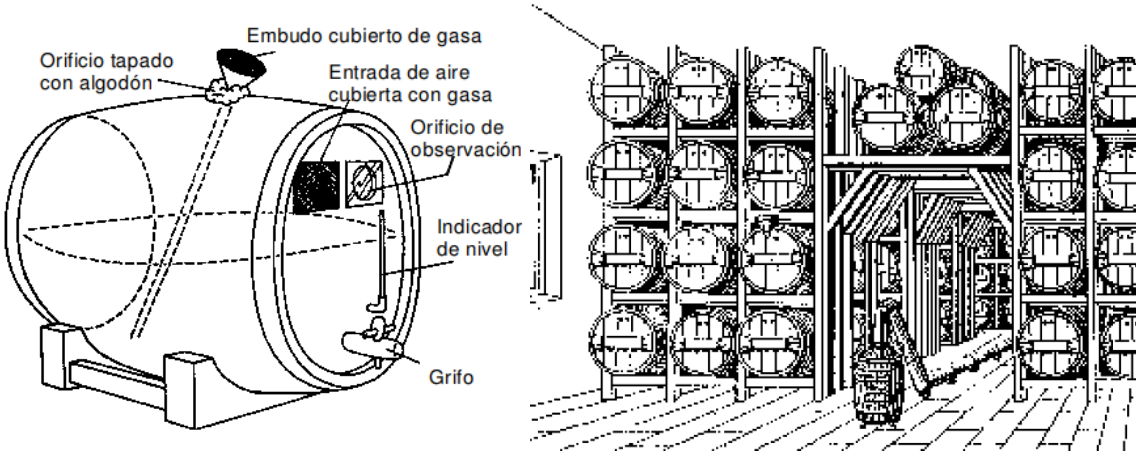


Figura 2. Esquema de una cuba de producción de vinagre por el método de cultivo superficial Orleans y pila de toneles para la acetificación por dicho método en una fábrica de vinagre. Fuente: (Polo, Sanchez-Luengo, 1991).

Aunque la mayoría de países utilizan el cultivo sumergido para la elaboración de vinagres, ya que es más rápido y económico, en el caso de los vinagres de vino de alta calidad, como son aquellos protegidos bajo una denominación de origen, se utilizan los métodos tradicionales, lentos, mediante cultivo superficial (Polo, Sanchez-Luengo, 1991).

Un aspecto importante que contribuye a la calidad organoléptica de los vinagres es el envejecimiento, que permite la incorporación o formación de nuevos compuestos con impacto sensorial. El aumento en la calidad organoléptica durante el envejecimiento es notable. Además de las interacciones con la madera, se producen una serie de reacciones químicas como la evaporación, la producción de ésteres por reacciones entre ácidos y alcoholes residuales, así como otros procesos, dando como resultado la presencia de nuevos aromas y metabolitos y una reducción en la sensación punzante del ácido acético (Callejón et al., 2018). Finalmente, una vez obtenido el vinagre por cualquiera de los métodos descritos, y tras un periodo de envejecimiento, el vinagre se estandariza y filtra.

1.1.2. NORMATIVAS ESPECÍFICAS VIGENTES SOBRE REGULACIÓN DE VINAGRES

El vinagre, su producción y comercialización, está regulado por diferentes estándares, e incluso la propia definición legal varía de un país a otro (Solieri & Giudici, 2009). La normativa regional Europea del Codex para el vinagre data del 1987 (CODEX-STAN-162, 1987), la cual define al vinagre como "líquido, apto para el consumo humano, producido exclusivamente con productos idóneos que contengan almidón o azúcares, o almidón y azúcares por el procedimiento de doble fermentación, alcohólica y acética". Además, en ella se describen diferentes tipos de vinagre, composición esencial y criterios de calidad junto con ingredientes opcionales, contaminantes, higiene, pesos y medidas, así como métodos de análisis. Aunque se han hecho varios intentos para convertir esta normativa regional europea en un estándar mundial, hasta ahora no se ha abordado esta conversión, debido a ciertos patrones comerciales y a diferencias regionales significativas. Esta norma regional no ha sido adoptada por todas las legislaciones nacionales de los Estados miembros debido a que en dos Estados el nombre "vinagre" se aplica al producto obtenido por dilución de ácido acético sintético, así como a los vinagres obtenidos de la fermentación de productos agrícolas. Por ello, en la normativa se ha incluido un nuevo título en la categoría de vinagres, siendo ahora "Vinagres y ácido acético diluido (diluido con agua hasta 4-30% en volumen) (COMMISSION REGULATION (EU) 2016/263).

En concreto en España, el Real Decreto 661/2012 recoge una actualización de la norma en la que dice que con el fin de garantizar la leal competencia entre las industrias, mejorar la competitividad del sector y dotar de las mismas condiciones a todos los productores, se necesita un marco normativo unitario, aplicable a todo el territorio nacional, y por tanto propone una adecuación de la normativa disponible sobre vinagres a la realidad del mercado, fundamentalmente, en lo relativo a la definición de nuevos productos, a las características de los productos terminados y a su etiquetado. Sin embargo, según esta normativa, el vinagre se define como "el líquido apto para el consumo humano resultante de la doble fermentación alcohólica y acética de productos de origen agrario". Por tanto, en España se sigue sin considerar "vinagre" a los producidos por dilución del ácido acético sintético.

Además, otra diferencia en cuanto a la normativa ocurre entre Europa y EE. UU. Así, en EEUU, la FDA (Administración de Drogas y Alimentos) exige que los productos de vinagre contengan al menos un 4% p/v de ácidos, sin especificarse estándares de identidad. Sin embargo, en la UE, el Reglamento (CE) 1493/1999 (Council Regulation, 1999), actualmente establece umbrales de acidez y alcohol residual, siendo la acidez mínima del 5% (p/v) y un máximo de 0.5% (v/v) de etanol, excepto para los vinagres de vino cuya acidez tiene que ser de al menos un 6% p/v y la cantidad máxima de etanol de 1.5% (v/v).

Por otro lado, tres programas de la UE sobre indicaciones geográficas y especialidades tradicionales, conocidos como Denominación de Origen Protegida (DOP), Indicación Geográfica Protegida (IGP) y Especialidad Tradicional Garantizada (TSG), se encargan de promover y proteger los nombres de los productos agrícolas y alimentos de calidad diferenciada. Los productos registrados bajo cualquiera de estas tres indicaciones se marcan con el logotipo correspondiente para ayudar a su identificación y diferenciación del resto. Estos están recogidos en el Reglamento nº 1151/2012 del Parlamento Europeo y del Consejo del 21 de noviembre de 2012 sobre los regímenes de calidad de los productos agrícolas y alimentarios (Regulation EU 1151, 2012), el cual es aplicado en la UE, aunque se está expandiendo gradualmente a nivel internacional mediante acuerdos bilaterales. Respecto a los vinagres, actualmente hay cinco DOP: tres de España (Vinagre de Jerez, Vinagre de Condado de Huelva y Vinagre de Montilla-Moriles) y dos de Italia (Aceto Balsamico Tradizionale de Modena y Aceto Balsamico Tradizionale di Reggio Emilia). Además, existe otro tipo de vinagre italiano denominado “Aceto Balsamico di Modena” que está registrado como una IGP.

1.1.3. VINAGRES DE VINO TRADICIONALES: DENOMINACIONES DE ORIGEN PROTEGIDAS

Entre los vinagres de vino tradicionales, con denominación de origen protegida, este trabajo de tesis se centra en los vinagres españoles con DOP: Vinagre de Jerez, el Vinagre de Condado de Huelva y el más recientemente añadido a las DOP, el Vinagre de Montilla-Moriles. Estos vinagres son de producción española, y más concretamente, su producción se centra en Andalucía.

Desde tiempos inmemoriales, Andalucía ha producido excelentes y afamados vinos y vinagres, sin los cuales no se entendería buena parte de la identidad y la cultura de esta región. Ya hace 2000 años, la Bética era la primera provincia productora de vino del imperio romano. A la historia del vino ha estado unido ineludiblemente el vinagre; primero, como un accidente en el proceso de vinificación, hasta convertirse posteriormente en un producto con personalidad propia y muy apreciado en todo el mundo, que posee múltiples aplicaciones culinarias. Precisamente, la singularidad y las características únicas de los vinos y vinagres andaluces han dado lugar al reconocimiento de tres DOPs: Vinagre de Jerez, reconocida en 1995, Vinagre del Condado de Huelva, reconocida en 2002, y la DOP Vinagre de Montilla-Moriles, reconocida en 2008. Estas tres denominaciones andaluzas de vinagres están estrechamente vinculadas a las denominaciones de origen protegido de vinos de igual nombre geográfico. La zona geográfica de cada una de estas DOP de vino proporciona la materia prima que necesitan sus respectivos vinagres, compartiendo Consejo Regulador y enmarca las regiones coloreadas en la Figura 3.

La Zona de Producción del Vinagre de Jerez o zona geográfica delimitada en la que se encuentran los viñedos inscritos de los que procede la uva para la elaboración de los vinos de partida enmarca los terrenos marcados en amarillo en la Figura 3, situados en los términos municipales de Jerez de la Frontera, El Puerto de Santa María, Sanlúcar de Barrameda, Trebujena, Chipiona, Rota, Puerto Real y Chiclana de la Frontera.

La Zona de Producción que acoge la DOP Vinagre del Condado de Huelva engloban la zona geográfica marcada en rojo en la Figura 3, en el que se encuentran los términos municipales de Almonte, Beas, Bollullos Par del Condado, Bonares, Chucena, Gibraleón, Hinojos, La Palma del Condado, Lucena del Puerto, Manzanilla, Moguer, Niebla, Palos de la Frontera, Rociana del Condado, San Juan del Puerto, Trigueros, Villalba del Alcor y Villarrasa, extendiéndose por la llanura del bajo Guadalquivir lindando con el Parque Nacional de Doñana.

Por otro lado, la Zona de Producción del Vinagre de Montilla-Moriles se enmarca en el denominado Valle del Guadalquivir, señalado en marrón en la Figura 3, el cual engloba los

términos municipales de Montilla, Moriles, Doña Mencía, Montalbán, Monturque, Nueva Carteya, Puente Genil, Aguilar de la Frontera, Baena, Cabra, Castro del Río, Espejo, Fernán Nuñez, La Rambla, Lucena, Montemayor, Santaella, además de la localidad de Córdoba, delimitada al norte por el Canal del Guadalmellato.

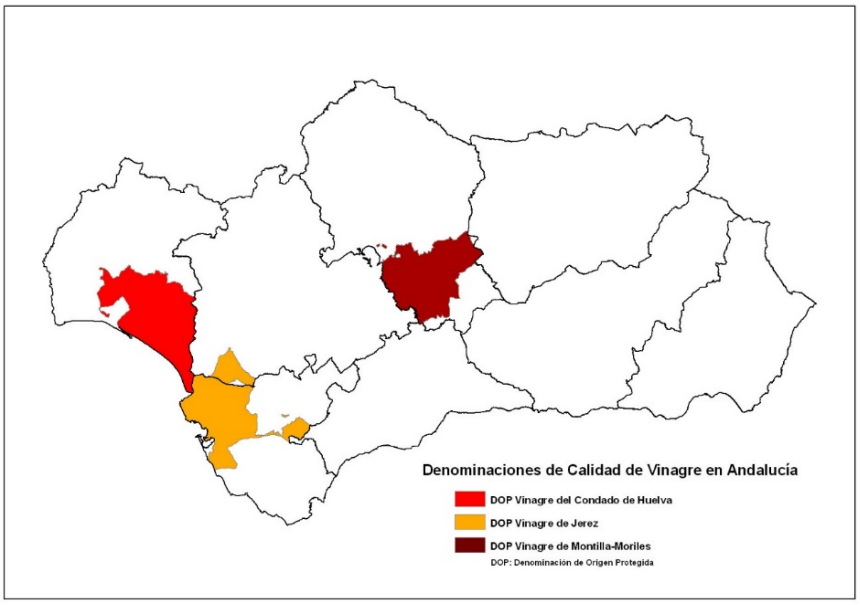


Figura 3. Zonas geográficas de producción amparadas bajo las denominaciones de origen protegidas de vinagres de vino.

Otra singularidad de los vinagres andaluces es su envejecimiento mediante el tradicional sistema de “criaderas y solera”, que les proporciona unas características organolépticas únicas y les abre una gran variedad de posibilidades de maridaje más allá del aderezo cotidiano. El reconocimiento de estos vinagres como DOP ha aportado un claro valor añadido a los vinagres originarios de estas zonas productoras, constituyendo una herramienta clave para ser competitivos en los mercados internacionales y para diversificar la producción vitivinícola.

1.1.3.1. Vinagre de Jerez

El Vinagre de Jerez es el producto resultante de la fermentación acética de vinos aptos elaborados en la "Zona de Producción" establecida, producido y envejecido mediante prácticas tradicionales y que reúne las características organolépticas y analíticas específicas que se describen en la **Tabla 2.**

Tabla 2. Características analíticas específicas de los vinagres protegidos bajo la DOP Vinagre de Jerez según su reglamentación específica (BOJA 15/10, 2008a).

Contenido	Límites	Excepciones/especificaciones
Alcohol residual	≤3% en volumen	Excepto en los vinagres al Pedro Ximénez o al Moscatel, en los que no debe superar el 4% en volumen
Acidez total en acético	≥70 g/L	Excepto en vinagres al Pedro Ximénez o al Moscatel, que podrá superar los 60 g/L y en Gran Reserva, con acidez total mínima de 80 g/L
Extracto seco	≥1,30 g/L y grado acético	Excepto en la categoría Gran Reserva con un mínimo de 2,30 g/L y grado acético
Cenizas	Entre 2 y 7 g/L	Excepto la categoría Gran Reserva que debe tener entre 4 y 8 g/L
Sulfatos	≤3,50 g/L	
Materias reductoras de los vinos utilizados		En las categorías al Pedro Ximénez o al Moscatel un mínimo de 60 g/L

La materia prima para la obtención de los vinagres de Jerez son los denominados "vinos aptos", que son aquellos vinos procedentes de bodegas situadas en la "Zona de Producción de Vinagre", que coincide con la zona de producción correspondiente a las Denominaciones de Origen "Jerez-Xérès-Sherry» y «Manzanilla — Sanlúcar de Barrameda". Estos vinos pueden ser los vinos del año expedidos a su graduación alcohólica volumétrica natural o los vinos criados que hayan cumplido con los períodos de envejecimiento promedio mínimo establecidos en sus correspondientes Pliegos de Condiciones. Las variedades de uva autorizadas para elaborar los vinos usados para producir el Vinagre de Jerez son la Palomino de Jerez, Palomino Fino, Moscatel y Pedro Ximénez, todas ellas blancas. De todas ellas, la Palomino es la variedad más característica del Marco de Jerez, con más del 95% de la superficie.

Figura 4. Sello de la DOP “Vinagre de Jerez”.

Para su producción, tras la desnaturalización del vino apto en el momento de su entrada en la bodega y su posterior acetificación, se procede a su envejecimiento. Todos los vinagres amparados bajo esta denominación tienen que tener al menos un periodo de envejecimiento igual o superior a seis meses en el caso del envejecimiento por el sistema de “criaderas y solera” (Figura 5).

Figura 5. Esquema del sistema de envejecimiento de “criaderas y solera”.

En el sistema de “criaderas y solera” tiene lugar la acetificación y el envejecimiento de forma simultánea. Consta de un número indeterminado de botas de madera agrupadas en filas horizontales o escalas (entre 3 o cinco), llamándose “solera” la fila que queda a ras de suelo, mientras que a la inmediatamente superior a ella se le llama “primera criadera”, a la siguiente “segunda criadera” y así sucesivamente. El envejecimiento va de menor a mayor, siendo la fila “solera” la que contiene los vinagres más envejecidos y de la cual se obtiene el vinagre elaborado. Una vez sacado una cierta cantidad de vinagre de esta “solera” (menor a un tercio de la bota), se repone con contenido de la “primera criadera” (Figura 5). El volumen de vinagre

extraído de la "primera criadera" se repone a su vez, con vinagre de la "segunda criadera", y así sucesivamente. La extracción de vinagre se denomina "saca" y la adición del mismo "rocío". Estas "sacas" y "rocíos" son realizados normalmente tres o cuatro veces al año.

Según los periodos de envejecimiento a los que son sometidos los vinagres de la DOP Vinagre de Jerez, que son aproximados debido a las características del envejecimiento en soleras, se distinguen las diferentes categorías que se recogen en la Figura 6. Adicionalmente, esta denominación incluye dos tipos de Vinagres de Jerez semi-dulces, en función del vino de la variedad correspondiente que se utilice en su producción: Vinagre de Jerez al Pedro Ximénez y Vinagre de Jerez al Moscatel. Éstas, a su vez, pueden someterse a un proceso de envejecimiento, y corresponder a cualquiera de las categorías de envejecimiento descritas.

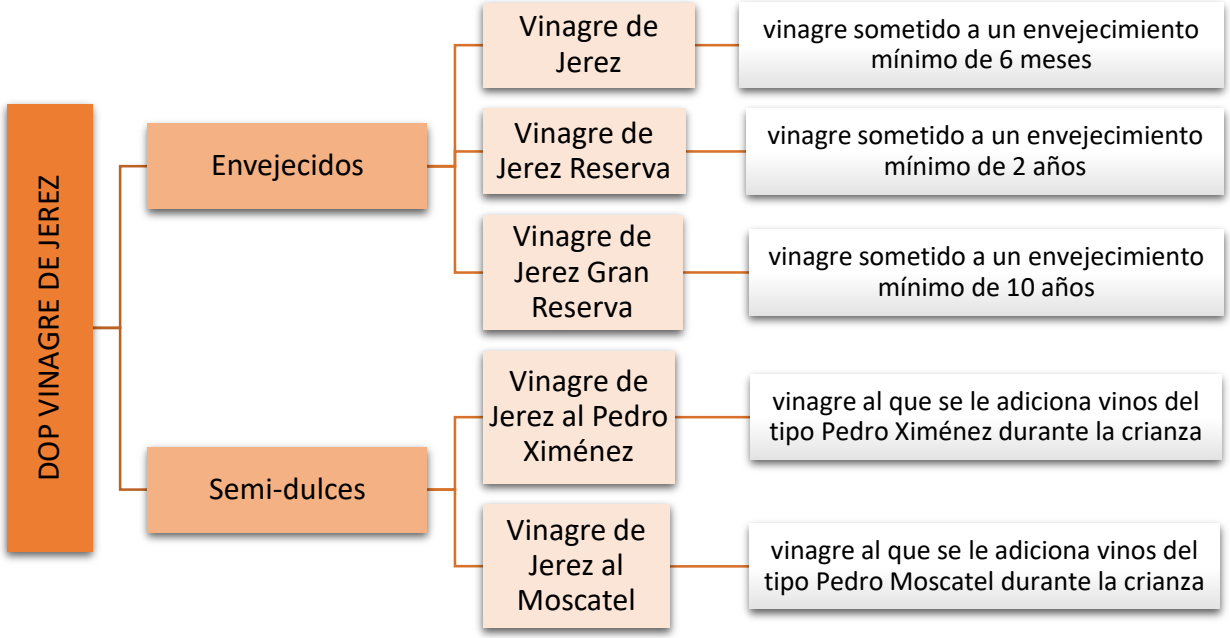


Figura 6. Esquema de las categorías establecidas para la DOP "Vinagre de Jerez".

1.1.3.2. Vinagre del Condado de Huelva

El Vinagre del Condado de Huelva es un vinagre de vino, procedente de la fermentación acética de un vino certificado por el Consejo Regulador de la Denominación de Origen "Condado de Huelva". Para la elaboración del vinagre amparado bajo esta denominación, la materia prima debe ser un vino blanco o generoso de la Denominación de Origen Protegida "Condado de Huelva", cuya zona de producción coincide exactamente con la zona geográfica de la DOP Vinagre del Condado de Huelva, y cuyas características son debidas a la variedad autóctona Zalema, propia y exclusiva de la zona geográfica delimitada. Por tanto, dicho vino utilizado proviene exclusivamente de la misma área geográfica definida para el vinagre.

Figura 7. Sello de la DOP "Vinagre del Condado de Huelva".

Respecto a las características sensoriales de estos vinagres amparados bajo la DOP Vinagre del Condado de Huelva, presentan colores y aromas que van desde el amarillo pálido o ámbar con aromas acéticos con toques de vino en los menos envejecidos, al color caoba y notas aromáticas de higos secos, pasas y madera en los más envejecidos. Los valores resultantes del examen analítico de los vinagres protegidos deberán estar incluidos dentro de los siguientes límites recogidos en la **Tabla 3.**

Tabla 3. Características analíticas específicas de los vinagres protegidos bajo la DOP "Vinagre del Condado de Huelva" según su reglamentación específica (BOJA 15/10, 2008b).

Contenido	Límites
Alcohol residual	≤ 0,5% vol. en vinagre sin envejecer ≤ 3% vol. en la categoría Vinagre Viejo
Acidez total en acético	≥ 70 g/L
Extracto seco soluble	≥ 1,30 g/L y grado acético
Cenizas	Entre 1 y 7 g/L
Acetoína	≥ 100 mg/L
Prolina	≥ 300 mg/L
Mercurio	≤ 0,05 ppm
Arsénico	≤ 0,5 ppm
Plomo	≤ 0,5 ppm
Cobre y Zinc	10 mg/L
Hierro	≤ 10 mg/L
Sulfatos	≤ 2g/L
Cloruros	≤ 1 g/L
Claridad (L*)	≥ 93%
Intensidad de color por croma (Cab)	≥ 20 unidades

Los vinagres producidos en el Condado de Huelva se engloban en dos tipos (Figura 8). Un primer tipo, que procede de la fermentación acética de un vino blanco o generoso de la DOP "Condado de Huelva" mediante el método industrial de fermentación sumergida, siendo la única de las 3 DOP que comercializa vinagres de vino sin envejecer; y un segundo tipo, denominado Vinagre Viejo, que se elabora a partir del Vinagre del Condado de Huelva que, a su vez, dependiendo del tiempo y del método utilizado para el envejecimiento se distinguen tres subtipos, Vinagre Viejo Solera, Vinagre Viejo Reserva y Vinagre Viejo Añada. De estos últimos, los vinagres Solera y Reserva son obtenidos por el envejecimiento tradicional de "criaderas y solera", anteriormente descrito para la DOP Vinagre de Jerez, pero con la singularidad de que durante todo el envejecimiento hasta la "saca" se adiciona a los vinagres con el tipo de vino Generoso o Generoso de Licor de la Denominación de Origen Condado de Huelva, mejorando el buqué del vinagre por formación de ésteres y nutriendo a las bacterias acéticas del alcohol procedente de estos vinos, de forma que no degraden el ácido acético ya formado.

El Vinagre Viejo Añada, a diferencia de Vinagre Viejo Solera y Vinagre Viejo Reserva, se envejece de forma estática por el tradicional método de “Añadas” durante un periodo mínimo de envejecimiento de treinta y seis meses. Aquí se deja el vinagre en envejecimiento de forma estática en las botas, pudiendo adicionar sólo vino Generoso o Generoso de licor durante el proceso de envejecimiento. Estos vinagres proceden de vinos de una sola añada, ya que no se realizan mezclas, y las características son intrínsecas de la añada en cuestión.

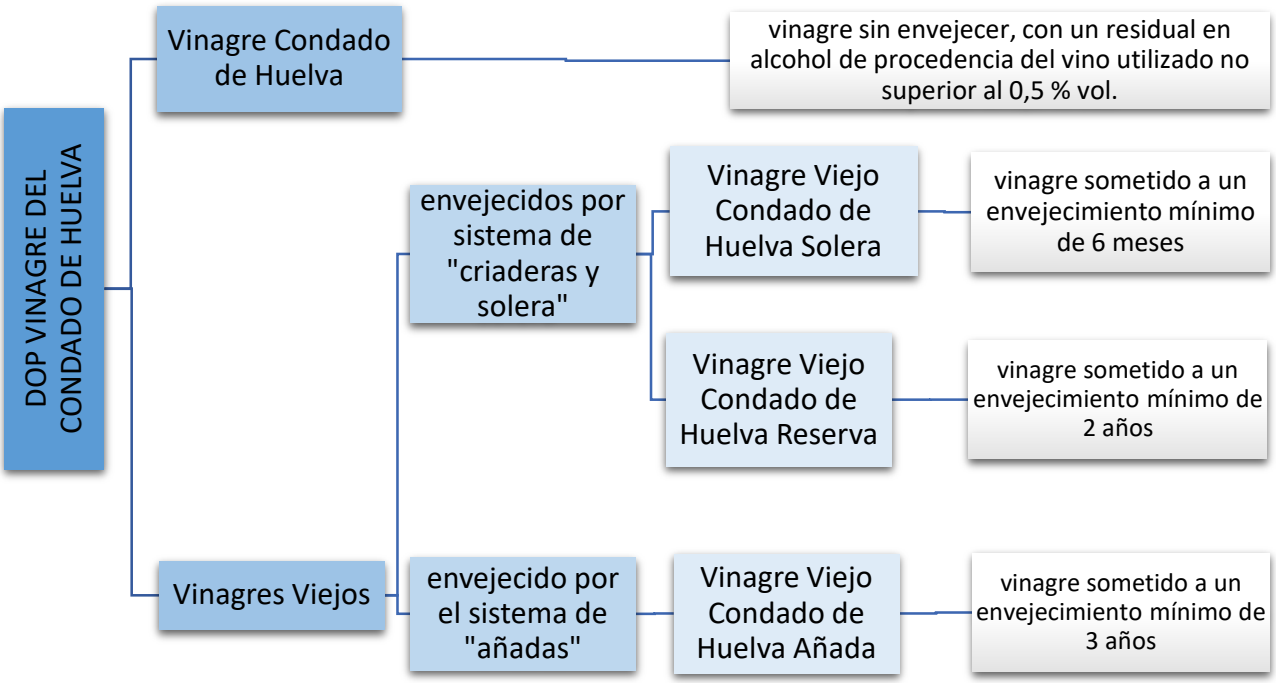


Figura 8. Esquema de las categorías establecidas para la DOP “Vinagre del Condado de Huelva”.

1.1.3.3. Vinagre de Montilla-Moriles

El "Vinagre de Montilla-Moriles" es un vinagre de vino obtenido exclusivamente de la fermentación acética de vinos de crianza certificado de la DOP "Montilla-Moriles", es decir, que provienen de la zona amparada por la DOP, o en su caso, un vinagre procedente de vinagre de vino obtenido de la fermentación acética de vino certificado de la DOP "Montilla-Moriles" con adición de mostos igualmente certificados de dicha denominación vínica, y sometido a envejecimiento. En 2008, por Orden de la Consejería de Agricultura y Pesca (BOJA 03/11, 2008) se emitió una decisión favorable en relación con la solicitud de inscripción de la Denominación de Origen Protegida "Vinagre de Montilla-Moriles", que cuenta con protección nacional desde el año 2009, siendo la última DOP de vinagre de vino española aceptada. Las características analíticas de los vinagres protegidos bajo esta denominación son las recogidas en la siguiente Tabla 4. Características analíticas específicas de los vinagres protegidos bajo la DOP "Vinagre de Montilla-Moriles**Tabla 4.**

Tabla 4. Características analíticas específicas de los vinagres protegidos bajo la DOP "Vinagre de Montilla-Moriles" según su reglamentación específica (BOJA 03/11, 2008).

Contenido	Mínimo o máximo	Excepciones
Alcohol residual	≤ 3% vol.	
Acidez total en acético	≥ 60 g/L	
Extracto seco soluble	≥ 1,30 g/L y grado acético	
Cenizas	Entre 2 y 7 g/L	En los vinagres dulces entre 3 y 14 g/L
Acetoína	≥ 100 mg/L	
Azúcares reductores	≥ 70 g/L en vinagres dulces	

La materia prima para la elaboración del "Vinagre de Montilla-Moriles" es el vino certificado por la DOP de vino "Montilla-Moriles". Los tipos de vino utilizados son Fino, Amontillado, Oloroso y Pedro Ximénez, con contenido alcohólico superior o igual al 15% vol. Las variedades de uva utilizadas, según el caso, son las variedades "Pedro Ximénez" o "Moscatel".

Los vinagres con envejecimiento presentan colores que van desde el ambarino hasta el color caoba casi azabache, con aromas suaves de ácido acético evolucionados con tonalidades de madera de roble. Tienen un sabor equilibrado y suave.

Figura 9. Sello de la DOP "Vinagre de Montilla-Moriles".

Los vinagres amparados bajo esta denominación son envejecidos a través del sistema tradicional de "criaderas y solera" y según el periodo de envejecimiento al que son sometidos pueden ser de "Crianza", "Reserva" o "Gran Reserva". También se incluyen vinagres envejecidos por el sistema estático "añada" por un tiempo igual o superior a tres años. Además, esta denominación presenta dos tipos de vinagres dulces, Vinagres al Pedro Ximénez y Vinagres al Moscatel, que poseen la peculiaridad de que para su producción se le adiciona mosto concentrado procedente de la uva pasificada de la variedad Pedro Ximénez y Moscatel, respectivamente, pudiendo utilizarse también, aunque con menor frecuencia, vinos dulces de la correspondiente variedad. Estos vinagres dulces a su vez pueden someterse a envejecimiento. Los tipos de vinagre amparados en esta DOP se resumen en la Figura 10.

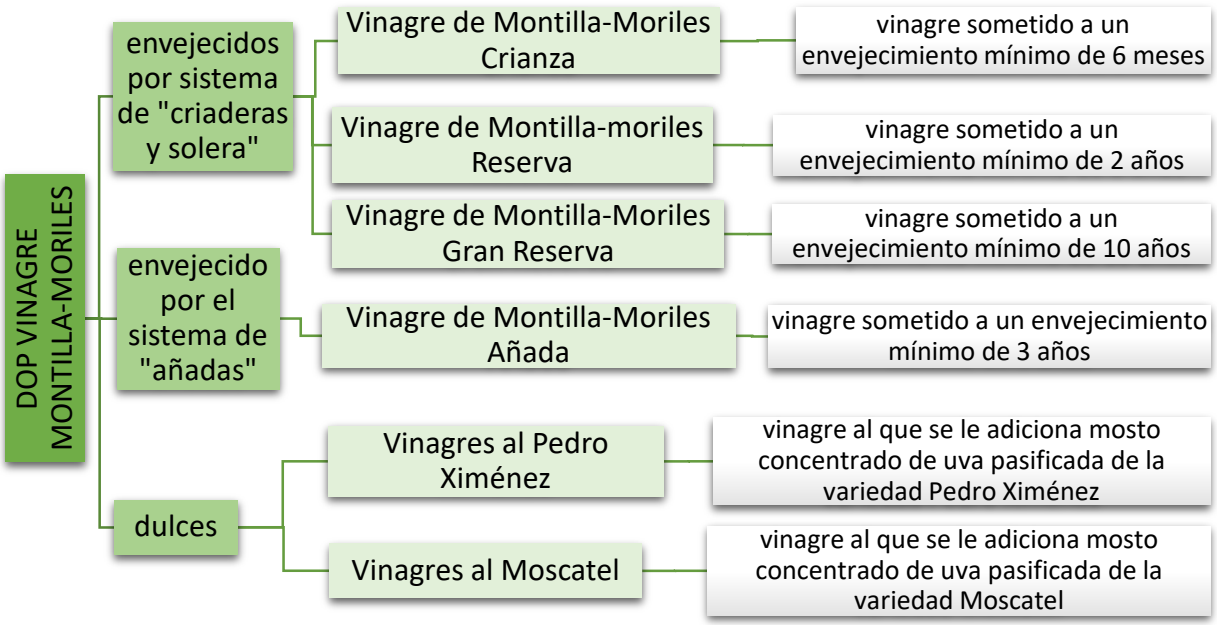


Figura 10. Esquema de las categorías establecidas para la DOP "Vinagre de Montilla-Moriles".

1.2. PARÁMETROS DE CALIDAD DEL VINAGRE DE VINO

En términos generales, los aspectos responsables de la calidad de un alimento más relevantes son el valor nutricional, la seguridad alimentaria y las propiedades sensoriales del producto. Sin embargo, como los vinagres de vino son principalmente usados como condimento, en su caso, la calidad está principalmente determinada por las propiedades sensoriales, y dentro de ellas, la calidad sensorial del vinagre se debe fundamentalmente a su aroma. Además del ácido acético y el etanol, el vinagre en general, así como especialmente el vinagre de vino, contiene otros constituyentes que desempeñan un papel importante en cuanto a su olor, sabor y cualidades conservadoras. Estos constituyentes que influencian al "flavor" del vinagre, son originados y están influenciados por el material de partida, el método de producción, los compuestos formados durante la fermentación, y en algunos casos, por los cambios que se producen durante el envejecimiento en madera (Tesfaye, Morales, Benítez, García-Parrilla, & Troncoso, 2004).

La materia prima proporciona una gran cantidad de compuestos relevantes para la calidad final del vinagre, como compuestos aromáticos característicos y polifenoles. Este último grupo de compuestos, que ejercen una fuerte influencia en las propiedades organolépticas (color, flavor y astringencia) y en propiedades beneficiosas para la salud, se encuentran en mayor cantidad en los vinagres de vino que en aquellos vinagres que se obtienen por ejemplo de la miel o de manzana (Cerezo et al., 2010).

El proceso de producción también tiene una gran influencia en la composición aromática del vinagre. De hecho, además del efecto en la composición aromática que tiene el método de acetificación empleado (sumergido o superficial), se ha demostrado también que la diversidad de especies de bacterias involucradas en la acetificación influye en la composición final del vinagre de vino (Tesfaye et al., 2002; Valero et al., 2005).

El envejecimiento en madera también contribuye en gran manera al incremento de la complejidad aromática de los vinagres de vino, influyendo también en el color de los mismos. Tanto el color como el aroma son características importantes para el consumidor a la hora de elegir un vinagre u otro, relacionándolos con la calidad de los mismos. Durante el envejecimiento en barricas de madera ocurren modificaciones químicas dentro de las que se incluyen la esterificación, la condensación y la concentración de compuestos debido a la evaporación de agua que se produce a través de los poros de la madera. Algunos compuestos también se extraen de la madera, lo que le confiere al vinagre final propiedades específicas y singulares (Callejón, Torija, Mas, Morales, & Troncoso, 2010; Marrufo-Curtido et al., 2012). El tiempo y tipo de

envejecimiento (en diferentes tipos de madera, etc.) son otras fuentes de variabilidad que afectan a la calidad del vinagre de vino.

La viscosidad es otro parámetro importante en la calidad sensorial de algunos vinagres de vino, especialmente en el caso del “Aceto Balsamico Tradizionale di Modena” o vinagre balsámico tradicional de Módena. Además, también afecta a la calidad de cualquier vinagre, la adición de extractos, azúcares, colorantes artificiales o conservantes, debido a que su presencia puede ser indicativo de una menor calidad.

1.3. FRAUDES Y PROBLEMAS DE AUTENTICIDAD DEL VINAGRE DE VINO

A continuación, se describen algunos fraudes o problemas de autenticidad comunes que pueden ocurrir en el vinagre de vino.

1.3.1. PROBLEMAS DERIVADOS DE LAS LEGISLACIONES NACIONALES E INTERNACIONALES

Debido a las diferencias observadas de un país a otro en la definición legal de “vinagre”, si un vinagre producido en un país se comercializa en otro en el que la definición de vinagre cambia, esto puede plantear un problema y riesgo para los consumidores, pudiendo convertirse en un problema de autenticidad si su origen no está claramente declarado. Por ejemplo, este problema ocurre entre Alemania y el resto de Europa. La definición legal alemana de "vinagre de vino" permite la producción de vinagre por fermentación acética a partir de etanol natural, diluyendo ácido acético con agua o mezclando vinagre de fermentación con ácido acético sintético, o con vinagre hecho de ácido acético sintético (Werner & Roßmann, 2015). Sin embargo, las regulaciones europeas indican que el vinagre de vino solo se puede producir a través de la fermentación acética del vino producido a partir de uvas frescas. Por lo tanto, comercializar "vinagre de vino", producidos con alcohol de diferentes orígenes, procedente de Alemania como vinagre de vino genuino en un país europeo, supondría un fraude para el consumidor. Además, la comercialización de vinagres producidos con alcohol de diferentes orígenes, como vinagre de vino genuino, es una de las actividades fraudulentas más comunes en la industria del vinagre. Esta práctica fraudulenta tiene como objetivo reducir los costos de fabricación y constituye un fraude para los consumidores. Esta adulteración es difícil de detectar debido a que a veces la procedencia del alcohol no es conocida (Sáiz-Abajo, González-Sáiz, & Pizarro, 2006).

1.3.2. PROBLEMAS Y FRAUDES RELACIONADOS CON LAS MATERIAS PRIMAS

1.3.2.1. Adición de ácido acético sintético

A lo largo de los años, se han descrito muchos fraudes en la industria del vinagre. Uno de los primeros fraudes que se conocen relacionados con el vinagre, y que ha estado ocurriendo durante más de ochenta años, es la adición de ácido acético sintético al vinagre, yendo esto en contra de las regulaciones de la industria del vinagre (Ríos-reina, Segura-Borrego, Úbeda, Morales, & Callejón, 2018). El vinagre obtenido por el ácido acético sintético se llama “vinagre de madera o esencia de vinagre”, y no se puede vender como vinagre fermentado porque contiene más metales pesados por kg de ácido acético puro que la cantidad permitida regulada (máximo 5 mg/kg de ácido acético puro) (Bourgeois, McColl, & Barja, 2006), lo que supone un riesgo para el consumidor y una práctica desleal para otros productores de vinagre. Por este motivo, la legislación europea indica que el vinagre de vino auténtico no puede contener ácido acético obtenido de derivados del petróleo o pirolisis de la madera (Bourgeois et al., 2006). La detección de la adición de ácido acético sintético al vinagre se ha realizado mediante la determinación de ácido fórmico, derivado de la pirolisis de la madera, ya que éste ha demostrado ser un indicador indirecto de ello (Bourgeois et al., 2006).

1.3.2.2. Adición de agua a uvas secas o a mosto concentrado

Otra práctica desleal relacionada con el vinagre de vino es producir el vinagre de vino, o el vino de partida, a partir de uvas secas diluidas con agua. Este producto llamado “vinagre de pasas” es producido comúnmente en algunos países mediterráneos mediante la fermentación de uvas secas y la rehidratación con agua, pese a no poder considerarse ni etiquetarse como “vinagre de vino”. No obstante, la producción de "vinagre de vino" por el método anterior se ha encontrado en algunos países mediterráneos como Grecia, siendo también importado incorrectamente en Italia como vinagre de vino. Debido a que este método reduce el precio de producción afectando a la genuinidad del producto, puede considerarse, en algunos países de Europa, como una actividad fraudulenta (Callejón et al., 2018; Camin et al., 2013; Ríos-Reina et al., 2018).

1.3.2.3. Adición de alcohol o azúcares no procedentes del vino

La comercialización de vinagres producidos con alcohol de origen distinto de las uvas como un vinagre de vino genuino, es una de las actividades fraudulentas más comunes en la industria del vinagre. Esta práctica tiene como objetivo reducir los costos de fabricación y supone un riesgo para la salud del consumidor. Otra de las prácticas desleales que se pueden dar es la adición de diferentes proporciones de vinagre de alcohol a las muestras de vinagre de

vino, lo que hace que el producto sea más barato de una forma fraudulenta, siendo también ésta una amenaza importante para el sector vinagrero. Estas adulteraciones son difíciles de detectar porque el alcohol agregado al vino de base antes del comienzo del proceso de fermentación no siempre tiene un origen botánico bien conocido (Sáiz-Abajo, González-Sáiz, & Pizarro, 2004a). De esta forma, el alcohol agregado a los vinagres de vino debe provenir de la fermentación de la piel de las uvas, y en ningún caso de otros materiales como la melaza, remolacha azucarera o caña de azúcar. Por lo tanto, el problema de autenticidad está relacionado con la dificultad de detectar cuál es la fuente del ácido acético del vinagre de vino. En el caso del vinagre balsámico como "Aceto Balsamico di Modena" IGP, también se puede dar la práctica desleal de agregar azúcares exógenos al mosto de uva cocido y/o concentrado (Callejón et al., 2018).

1.3.3. PROBLEMAS Y FRAUDES DEBIDOS A LA MEZCLA DE DIFERENTES VINAGRES

Otra práctica fraudulenta posible en la elaboración y comercialización del vinagre de vino es la mezcla de diferentes proporciones de vinagre de vino y vinagre de alcohol. El problema de autenticidad en este caso ocurre cuando esta mezcla se vende bajo la denominación de vinagre de vino, como si fuera un producto puro. Otro de los fraudes que han ocurrido durante largo tiempo en países como Suiza ha sido la adición de vinagre de sidra al vinagre de vino para reducir los costos de producción (Bourgeois et al., 2006). En general, un buen método para una diferenciación segura entre un vinagre de vino puro y uno mezclado es la identificación de ácidos de frutas específicos, aunque esto puede manipularse fácilmente con la adición de ácidos y aminoácidos específicos de frutas.

1.3.4. PROBLEMAS Y FRAUDES RELACIONADOS CON LAS INDICACIONES GEOGRÁFICAS

Los vinagres que se encuentran regulados con una indicación geográfica como la denominación de origen protegida son también objeto de fraude. Si bien la existencia de denominaciones de origen protegidas o etiquetas de calidad en los vinagres, muy comunes en el sur de Europa, ofrece una mayor garantía al producto, al mismo tiempo esto aumenta el valor añadido del producto pudiendo fomentar la posibilidad de fraude al poder venderse un vinagre sin DOP como si en realidad como si en realidad estuviera protegido por esta indicación geográfica. Aunque estas DOP regulan estrictamente su procedimiento de producción, el área geográfica de producción, el envejecimiento a través de las prácticas tradicionales y las características analíticas y organolépticas, aun así, pueden ocurrir ciertas adulteraciones o fraudes. Estos actos engañan al consumidor y crean una competencia desleal entre los productores principalmente debido al beneficio extra. Un caso conocido es el del vinagre balsámico tradicional de Módena con DOP y el vinagre balsámico de Módena IGP. El primero se

produce mediante un método de producción tradicional, costoso y que consume mucho tiempo y obedece a normas muy estrictas de procedencia de la materia prima y métodos de producción, lo que garantiza una alta calidad. El segundo es producido industrialmente y es un producto mucho más barato hecho de mosto cocido, mosto concentrado y vinagre de vino a través de un proceso complicado, pero mucho más rápido que el empleado para el vinagre balsámico tradicional, y la calidad del vinagre final es menor (Consonni et al., 2008a; Consonni, Cagliani, Rinaldini, & Incerti, 2008). Por tanto, la venta de un vinagre balsámico de Módena (IGP) como un vinagre tradicional balsámico de Módena (DOP) es considerado un fraude.

1.3.5. PROBLEMAS Y FRAUDES RELACIONADOS CON LOS PROCESOS DE PRODUCCIÓN Y ENVEJECIMIENTO

Otro tipo de adulteración se produce durante el proceso de producción, afectándose principalmente los vinagres producidos por sistemas tradicionales como el Vinagre de Jerez o el Vinagre Balsámico Tradicional de Módena. Debido a que los vinagres producidos por métodos tradicionales están asociados con una mayor calidad, junto con un mayor costo y tiempo de producción, en comparación con los producidos por métodos rápidos, existe un mayor interés en falsificar estos primeros y, por otro lado, existe también una mayor preocupación en encontrar un método capaz de diferenciarlos y evitar estos fraudes en el mercado.

Otro problema de autenticidad surge cuando hay un tiempo de envejecimiento mínimo especificado para un vinagre en particular, como en el caso de distintas categorías establecidas dentro de la DOP Vinagre de Jerez, Vinagre de Condado de Huelva o Vinagre de Montilla-Moriles, así como en el Vinagre Balsámico Tradicional de Módena, el cual se comercializa después de un proceso de envejecimiento de al menos 12 años en barrica (Consonni et al., 2008b). Las propiedades organolépticas del vinagre que se desarrollan durante el envejecimiento hacen que el producto final sea muy atractivo ya que aumenta su riqueza sensorial. Sin embargo, el tiempo y el coste de producción son excesivos para permitir un comercio lucrativo, y es por ello por lo que existe una elevada vulnerabilidad del producto ya que se pueden cometer actos desleales, comercializando vinagres con menor envejecimiento, o incluso sin envejecer, etiquetados y a precio de los que sí lo están. En este contexto, uno de los objetivos de la industria del vinagre hoy en día es producir vinagres con las mismas características obtenidas gracias al envejecimiento, pero haciéndolo de manera más económica y rápida sin perder calidad. Por esta razón, la industria del vinagre tiene un elevado interés en acelerar el envejecimiento siempre que sea de una manera que no genere un producto inferior o que provoque que el consumidor sea engañado. Además, existe una creciente necesidad de desarrollar métodos simples capaces de detectar metabolitos específicos en los vinagres como posibles indicadores del proceso de

envejecimiento y los procedimientos tradicionales, para proteger a los consumidores y evitar competencias desleales (Callejón et al., 2018).

1.3.6. PROBLEMAS Y FRAUDES RELACIONADOS CON LA ADICIÓN DE CARAMELO DE MOSTO

La adición de caramelo de mosto en ciertos vinagres de vino está permitida con el fin de unificar e color final del vinagre. Sin embargo, también podría utilizarse para simular el color de un vinagre envejecido sin que haya sido envejecido en absoluto, cuando su reglamento marca la obligación de serlo, o haber sido envejecido por un menor período de tiempo, lo cual abarata costes, pero también reduce en gran medida su calidad final (Werner & Roßmann, 2015). Esta práctica inapropiada podría afectar especialmente a las DOP de vinagre de vino español en las que los periodos de envejecimiento están claramente definidos (Ríos-Reina et al., 2018).

1.4. MÉTODOS PARA LA CARACTERIZACIÓN, CLASIFICACIÓN Y AUTENTIFICACIÓN DE VINAGRES DE VINO

Hoy en día, la creciente diversidad de vinagres en el mercado y la creciente demanda de vinagres y condimentos de calidad han creado la necesidad de caracterizarlos, estableciendo parámetros específicos y proporcionando un control de calidad adecuado para defender su identidad (Cerezo et al., 2008; Liu et al., 2008; Marrufo-Curtido et al., 2012). Además, debido a lo anterior, estos productos se están convirtiendo en objetivos mayores para el fraude y requieren nuevas herramientas para combatir la falsificación o el etiquetado incorrecto. Y más relevancia tiene todo esto en aquellos vinagres de vino protegidos por una DOP, donde más allá del cumplimiento de ciertos parámetros y detalles, el cliente desea tener garantía de que la diferencia de precio se debe a que los estándares de calidad son más elevados. Por ello, la caracterización del vinagre persigue el objetivo de proteger a los consumidores contra la comercialización de productos de calidad inferior a la declarada en su descripción, así como de defender a los productores que aplican las buenas prácticas de aquellos que ejercen una competencia desleal. Por lo tanto, el vinagre, como todos los demás productos alimentario, debe cumplir con las especificaciones de calidad y debe llevar una etiqueta que describa fielmente el producto. Y para controlar todo ello se necesitan soluciones instrumentales que sean fiables, que puedan evaluar los requisitos de calidad a partir de parámetros objetivos y que puedan garantizar la veracidad de la información declarada en la etiqueta. Es decir, se necesitan procedimientos de autentificación robustos y eficaces.

1.4.1. MÉTODOS OFICIALES

Para evaluar la calidad y autenticidad de los vinagres, varios países han establecido métodos y rangos aceptables o valores de guía para evaluar algunos parámetros del vinagre, basados en los resultados obtenidos en el análisis de un gran número de muestras auténticas. Las directivas nacionales e internacionales actuales incluyen métodos diseñados para la identificación de vinagre de vino y, en general, para el control de la autenticidad. En esta sección, se describen los métodos reconocidos oficialmente que se utilizan regularmente para los vinagres de vino (**Tabla 5**).

Debido a la gran diversidad de tipos de vinagre producidos a partir de diferentes materias primas y por diferentes procesos de producción, existen en el mercado numerosos vinagres con diferentes calidades. Esto conlleva a una creciente necesidad de investigar métodos analíticos fiables que puedan determinar la calidad y el origen. Además de evaluar la autenticidad de un vinagre, estos métodos deben ser capaces de detectar posibles adulteraciones y fraudes. En general, además de los métodos oficiales, existen metodologías alternativas que se pueden agrupar en dos tipos: análisis sensoriales y análisis fisicoquímicos.

Tabla 5. Métodos oficialmente reconocidos por la Organización Internacional de la viña y el vino (OIV) para probar la autenticidad del vinagre de vino. *Nota: Tabla adaptada del capítulo de libro: Ríos-Reina et al., (2018). Vinegar. FOODINTEGRITY Handbook. https://doi.org/10.32741/fihb.* **(ANEXO I).**

MÉTODO	REFERENCIA	TÉCNICA	OBJETIVO
Determinación del contenido de acidez total	OENO 52/2000	Neutralización de ácidos por solución alcalina	Cumplir requisitos legales (definiciones, DOP, IGP ...)
Determinación del contenido de acidez total fija	OENO 53/2000	Neutralización de ácidos no volátiles por solución alcalina	Cumplir requisitos legales (definiciones, DOP, IGP ...)
Determinación del contenido en acidez volátil	OENO 54/2000	Diferencia entre acidez total y acidez fija, en gramos de ácido acético por litro	Cumplir requisitos legales (definiciones, DOP, IGP ...)
Detección y cuantificación de ácido acético de síntesis	OENO 55/2000	Extracción del ácido acético con sosa, medida de la radioactividad por centelleo líquido, transformado en benceno	Autentificación: Valores de ^{14}C menores a los característicos del año de producción significa: mezcla con productos de años más recientes, o adición de ácido acético sintético
Determinación del contenido en alcohol residual	OENO 56/2000	Destilación, oxidación del etanol por $K_2Cr_2O_7$ y valoración del exceso con $FeSO_4$ y NH_4	Cumplir requisitos legales (definiciones, DOP, IGP ...)
Determinación del contenido en extracto seco total	OENO 57/2000	Evaporación, secado en horno y pesada	Detección de fraudes: adición de agua o una solución acuosa de ácido acético (valor muy bajo) o adición de sustancias no volátiles (valor muy alto). Base de datos necesaria
Determinación del contenido en cenizas	OENO 58/2000	Incineración del extracto del vinagre hasta combustión completa del carbono	Detección de fraudes: adición de agua o un ácido acético acuoso (valores muy bajos) o adición de sustancias no volátiles (valores muy altos). Base de datos necesaria
Determinación del contenido en sustancias reductoras no volátiles	OENO 59/2000	Evaporación de sustancias volátiles, hidrólisis clorhídrica, oxidación por solución alcalina de Cu en exceso con titulación por yodometría de iones de Cu	Detección de fraudes: adición de sustancias no volátiles
Determinación del contenido en dióxido de azufre	OENO 60/2000 OENO 13/2008	Titulación yodométrica directa (SO_2 libre) y doble hidrólisis alcalina (SO_2 combinado)	Controlar el nivel de SO2 y verificar el cumplimiento de normas y requisitos
Determinación del contenido en ácido ascórbico total	OENO 61/2000	Oxidación del ácido ascórbico por yodo, precipitación, separación por cromatografía de película delgada y determinación colorimétrica a 500 nm	Detección de uso tecnológico fraudulento
Determinación del contenido en cloruros	OENO 62/2000	Titulación potenciométrica de iones Cl con nitrato de plata en ambiente ácido	Detección del aumento fraudulento del extracto seco mediante la adición de NaCl
Determinación del contenido en sulfatos	OENO 63/2000	Precipitación de sulfatos con cloruro de bario, secado, calcinación y pesada	Detección de fraudes con objeto de aumentar el extracto seco

Determinación del contenido en cobre, zinc y hierro	OENO 64-65-66/2000	Medida directa por espectrofotometría de absorción atómica	Contaminación por materiales de contacto durante la fabricación, y el propio contenido del vino de partida. Un contenido excesivo puede causar alteraciones graves en el color
Determinación del contenido en plomo	OENO 67/2000	Medida directa por espectrometría de absorción atómica sin llama (atomización electrotérmica).	Contaminación de los materiales de contacto durante su fabricación, y el plomo del propio vino de partida
Determinación del contenido en mercurio	OENO 68/2000	Mineralización, reducción por MnO_4^- y medida por espectrometría de absorción atómica (vapor frío)	Problemas toxicológicos
Determinación del contenido en acetoína	OENO 69/2000	Neutralización a pH 7 con $Ca(OH)_2$. Medición directa por cromatografía de gases (GC)	Autentificación: determinación de la calidad y el origen mediante el contenido de acetoína en vinagres de vino (entre 100 mg/L y 400 mg/L)
Determinación del metanol, de los alcoholes superiores y acetato de etilo	OENO 70/2000	Neutralización a pH 7 con NaOH. Medición a través de GC de volátiles como etanol, propan-1-ol, butan-2-ol, butan-1-ol y 2-metilbutan-1-ol y 3-metilbutan-1-ol	Cuestiones organolépticas y posiblemente toxicológicas
Autentificación por SNIF-RMN y otros métodos isotópicos	OENO 71/2000	Extracción del ácido acético con éter. Purificación y determinación de su pureza. Medición de la relación $^2H/H$ a través de ^2H-RMN	Detección de fraudes sobre presencia de ácido acético sintético y detección de la adición de alcohol de plantas con metabolismo C4 (azúcar de la caña) o C3 (remolacha)
Detección del ácido acético de síntesis por la determinación de la radioactividad β del ^{14}C del ácido acético por centelleo líquido	OENO 12/2006	Extracción del ácido acético del vinagre. Valor de emisión β del ^{14}C comparado con el promedio de las emisiones β del ^{14}C en el etanol en vinos genuinos de cosecha tardía	Detección de fraudes sobre la adición de ácido acético sintético y control del año de elaboración de los vinos crudos
Determinación de la relación isotópica $^{13}C/^{12}C$ del ácido acético por espectrometría de masas isotópicas	OIV-OENO 510-2013	Proporción de isótopos $^{13}C/^{12}C$ de ácido acético por espectrometría de masas de isótopos (IRMS)	Detección de fraudes sobre el origen botánico del ácido acético y revelación de la adición de ácido acético sintético. Determinación de la adición de azúcar de caña.
Determinación de la relación isotópica $^{18}O/^{16}O$ del agua en el vinagre de vino por espectrometría de masas isotópicas	OIV-OENO 511/2013	Relación isotópica de $^{18}O/^{16}O$ de agua por IRMS	Detección de fraudes sobre la producción de vinagres a partir de uvas frescas o de uvas secas con adición de agua
Determinación de la distribución de ^2H en el ácido acético mediante RMN	OIV-OENO 527-2015	^1H-NMR y ^2H-SNIF-NMR	Detección de fraudes sobre el origen botánico del ácido acético y revelación de la adición de ácido acético sintético

1.4.2. MÉTODOS PARA EL ANÁLISIS SENSORIAL

Debido a que la calidad de un vinagre se asocia principalmente a su aroma, la evaluación sensorial es el primer método a tener en cuenta. El análisis sensorial ha demostrado ser una herramienta eficaz, simple y fiable para evaluar y apreciar la calidad del vinagre desde el punto de vista del productor, investigador o consumidor (Tesfaye et al., 2010). Sin embargo, el análisis sensorial en el caso del vinagre es particularmente arduo debido al sabor ácido y olor punzante del producto como consecuencia del ácido acético que contribuye de forma intensa a la sensación general. Por lo tanto, la metodología sensorial apropiada debe estar claramente definida y los atributos utilizados en el análisis discriminante o descriptivo deben ser precisos y bien reconocidos por el panel (Tesfaye et al., 2010).

La caracterización sensorial con el fin de controlar la calidad del vinagre de vino ha sido ampliamente utilizada durante muchos años (Callejón, Morales, Silva Ferreira, & Troncoso, 2008; Charles et al., 2000; González-Viñas, Salvador, & Cabezudo, 1996; Hillmann et al., 2012; Lalou et al., 2015; Tesfaye, García-Parrilla, 2002). Así, Gerbi et al. (1997) realizaron un análisis sensorial de vinagres de diferentes fuentes mostrando la capacidad del análisis sensorial para diferenciar vinagres de alcohol y vinagres de manzana de los vinagres de vino en base a sólo siete parámetros sensoriales (Gerbi, Zeppa, Antonelli, & Carnacini, 1997). Algunos años más tarde, Tesfaye et al. (2002) desarrollaron una evaluación sensorial de los vinagres de vino de la DOP de Jerez según los cambios que ocurrieron durante el envejecimiento. Este estudio mostró claramente que tanto la intensidad del aroma como la calidad aumentaban con el envejecimiento y se podía percibir a nivel sensorial (Morales, Tesfaye, García-Parrilla, Casas, & Troncoso, 2002). Morales et al. (2006) también estudiaron la importancia del perfil sensorial de los vinagres de vino producidos por el envejecimiento acelerado en comparación con los elaborados por un método tradicional y, por lo tanto, la capacidad de diferenciar un vinagre "rápido" de un vinagre de vino de alta calidad a nivel sensorial (Morales et al., 2006).

Gran parte de las investigaciones tradicionales sobre el control de la calidad del vinagre se han basado en una serie de análisis sensoriales. Además, en algunos vinagres, como es el caso del vinagre balsámico tradicional de Módena, su control de calidad se basa principalmente en el análisis de sus propiedades sensoriales. El análisis sensorial del vinagre se puede realizar mediante análisis olfativo y gustativo, así como mediante la determinación de otros parámetros como la viscosidad y el color.

1.4.2.1. Olor y sabor

Para analizar el sabor y el olor de los vinagres de vino, existen diferentes protocolos, pudiéndose realizar análisis gustativos y olfativos. A nivel gustativo, existen diferentes metodologías entre las cuales se encuentra la de preparar el vinagre de la forma que se asemeja más al procedimiento habitual de consumo, usando hojas de lechuga como matriz a la que se le adiciona vinagre (González-Viñas et al., 1996) o diluyendo el vinagre con agua fría o caliente. Otro método consiste en la evaluación directa del sabor y aroma del vinagre tal y como es, usando tazas opacas para evitar influencias de color, siendo este el análisis sensorial habitual realizado en las bodegas de vinagre (Tesfaye, García-Parrilla, 2002).

Dentro de los diferentes tipos de análisis sensorial de las características olfativas, los más utilizados son la prueba descriptiva, útil para determinar el perfil sensorial de las muestras, y la prueba discriminatoria, que incluye una amplia gama de pruebas como la prueba triangular (ISO.4120, 1983), pruebas de comparación pareadas (ISO.5495, 1983), y las pruebas de preferencia o hedónicas. Estos métodos requieren un panel sensorial entrenado y unos atributos definidos, concretos y adecuados.

1.4.2.2. Color

El color es uno de los parámetros más importantes utilizados por los consumidores para evaluar la calidad de un producto alimentario. Algunos estudios centrados en los vinagres de vino han descrito una relación entre algunos compuestos y un color más oscuro, como la presencia de melanoidina o de productos de la degradación de los azúcares y reacciones de Maillard (Solieri & Giudici, 2009). Un color más oscuro también se ha relacionado con un período de envejecimiento más largo en vinagres de vino y vinagre balsámico tradicional de Módena. Algunas técnicas como la espectrofotometría UV-Visible, la fluorescencia de emisión-excitación o las técnicas colorimétricas de transmisión se están utilizando para analizar el color, obteniéndose resultados satisfactorios (De la Haba, Arias, Ramírez, López, & Sánchez, 2014; Palacios, Valcarcel, Caro, & Perez, 2002; Zhu, Ji, Eum, & Zude, 2009). Sin embargo, el color podría modificarse fácilmente con el uso de caramelo de uva u otros aditivos, no habiéndose aún establecido oficialmente ningún método para evaluar y controlar este parámetro (Callejón et al., 2018).

1.4.3. MÉTODOS PARA EL ANÁLISIS FISICOQUÍMICO

El otro tipo de técnicas para estudiar en el campo de la caracterización y autentificación son aquellas que analizan las características fisicoquímicas de los vinagres. Aunque el análisis sensorial juega un papel importante en la aceptabilidad del vinagre desde el punto de vista del consumidor, y es el método que tradicionalmente se ha utilizado, se necesitan metodologías más rápidas y objetivas como son los métodos instrumentales, para garantizar la calidad final y comprobar el cumplimiento de los requisitos legales (Callejón et al., 2018).

Estas técnicas fisicoquímicas se pueden agrupar, a su vez, de acuerdo con dos estrategias: la primera consiste en aquellas técnicas capaces de analizar uno o más componentes específicos que podrían ser marcadores de un vinagre específico (métodos dirigidos o "targeted"), mientras que la otra estrategia está formada por técnicas que intentan obtener la "huella dactilar" o "fingerprint" (métodos no dirigidos o "untargeted"), o perfil de un vinagre analizado por una técnica y luego construyendo modelos de clasificación mediante el uso de herramientas quimiométricas (Cocchi et al., 2004). La Figura 11 muestra las etapas que conforman ambas estrategias para el análisis de vinagre.

Con respecto a la primera estrategia, los métodos convencionales ampliamente utilizados para caracterizar y autenticar vinagres mediante el análisis de uno o varios marcadores incluyen a los métodos oficiales anteriormente mencionados, así como la determinación de ciertos aminoácidos, de subproductos de la fermentación de *acetobacter* o sustancias derivadas de materias primas, y, en algunos vinagres, de los compuestos fenólicos derivados del envejecimiento en madera. La determinación y cuantificación de estos compuestos se han realizado mediante metodologías tales como la cromatografía de gases-espectrometría de masas (GC-MS) (Plessi, Bertelli, & Miglietta, 2006), la cromatografía líquida de alto rendimiento (HPLC-MS) (Callejón, Torija, Mas, Morales, & Troncoso, 2010; Cerezo et al., 2008; Tesfaye, Morales, García-Parrilla, & Troncoso, 2002), o métodos enzimáticos (Verzelloni, Tagliazucchi, & Conte, 2007).

La principal fortaleza de la segunda estrategia, la cual se basa en la determinación de un perfil o huella dactilar, consiste en tener en cuenta tanto la contribución individual como las interacciones de los diferentes componentes presentes en el vinagre, es decir, la complejidad total de la matriz alimentaria (Cocchi et al., 2004). En este caso, las metodologías estudiadas que permiten el desarrollo de esta estrategia son por ejemplo técnicas espectroscópicas, como la espectroscopía de infrarrojo medio y cercano (MIR, NIR) (De la Haba et al., 2014; Guerrero,

Mejías, Marín, Lovillo, & Barroso, 2010; Y. Zhao, Zhang, Zhao, Zhang, & Liu, 2011), espectroscopía de fluorescencia (Callejón, Amigo, Pairo, Garmón, et al., 2012) y resonancia magnética nuclear (RMN) (Fotakis, Kokkotou, Zoumpoulakis, & Zervou, 2013; Papotti et al., 2015); e incluso análisis no dirigido por GC-MS (Casale, Armanino, Casolino, Oliveros, & Forina, 2006a) estudiando el perfil volátil total de una muestra.

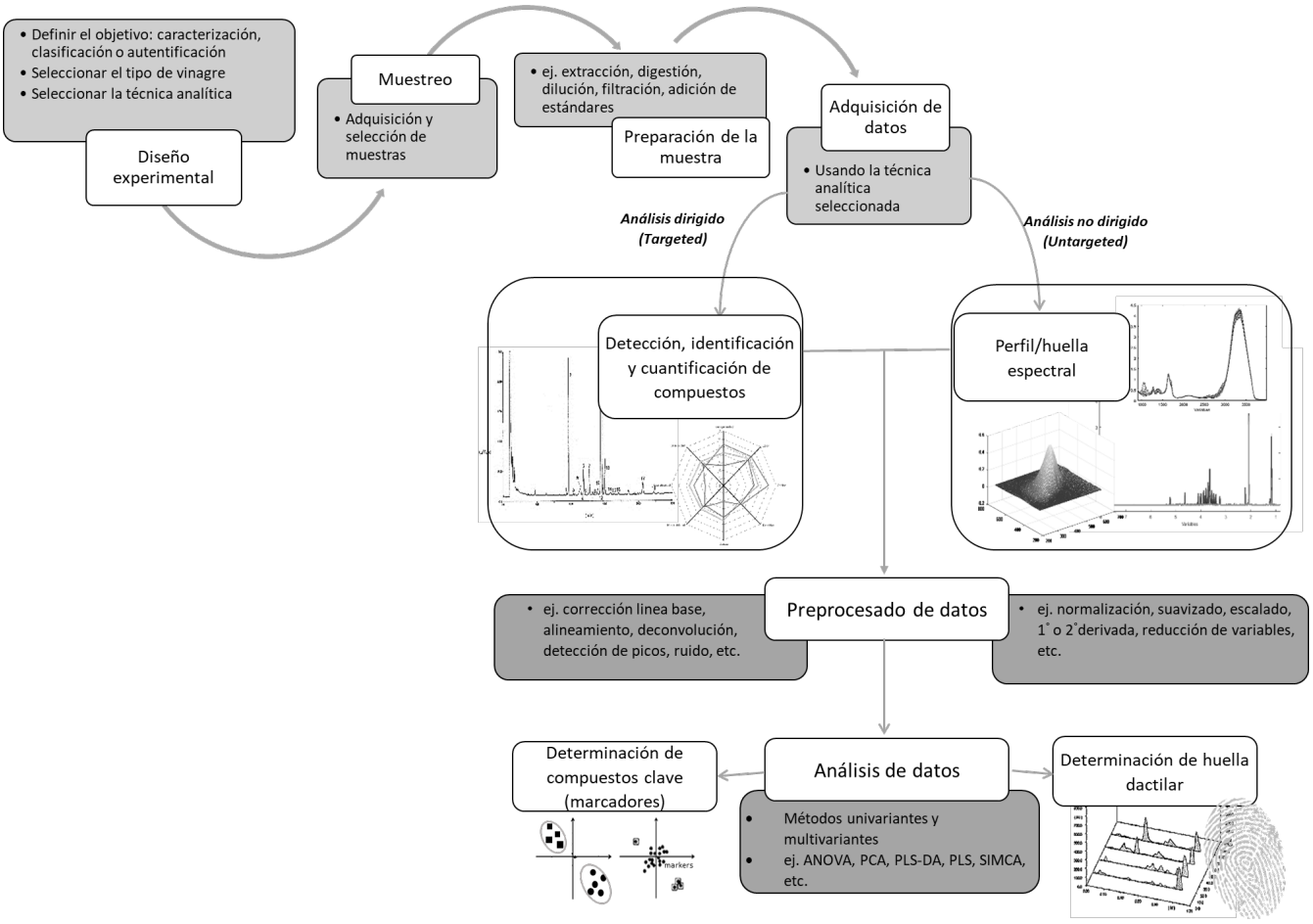


Figura 11. Etapas del procedimiento analítico dirigido y no dirigido para la caracterización, autentificación y clasificación de vinagres. Figura adaptada del capítulo de libro: *Ríos-Reina et al., 2018.* Fraud, quality and methods for characterization and authentication of vinegars*, BOOK "Advances in Vinegar Production"* **(ANEXO II).**

1.4.3.1. Técnicas cromatográficas

Las técnicas cromatográficas han sido aplicadas ampliamente y durante mucho tiempo para determinar ciertos compuestos del vinagre que presentan utilidad para su caracterización, clasificación o detección de adulteraciones en los vinagres. A continuación, se describen y discuten brevemente las técnicas empleadas en la presente memoria de tesis.

1.4.3.1.1. Determinación de compuestos volátiles y aromas

1.4.3.1.1.1. Técnicas de extracción

Desde un punto de vista analítico, el aroma del vinagre está formado por una compleja fracción de compuestos con un amplio rango de volatilidades, polaridades y concentraciones (Blanch, Tabera, Sanz, Herraiz, & Reglero, 1992). Por eso, aunque la cromatografía de gases (GC), o en concreto la cromatografía de gases-espectrometría de masas (GC-MS), es la técnica más utilizada para analizar la composición volátil del vinagre de vino, cuenta con la desventaja de necesitar normalmente un paso de extracción antes del análisis (Hantao et al., 2012; Marín, Zalacain, De Miguel, Alonso, & Salinas, 2005), fundamentalmente para poder determinar aquellos compuestos minoritarios presentes en bajas concentraciones.

Las técnicas de extracción de los compuestos volátiles del vinagre están basadas en distintas propiedades físico-químicas como son la volatilidad, la solubilidad en las distintas fases orgánicas inmiscibles con la matriz y la capacidad para ser adsorbidos selectivamente sobre ciertos materiales. Dentro de las técnicas que han sido aplicadas para la extracción del aroma del vinagre de vino cabe destacar: extracción por espacio de cabeza estático (HSE) (Ferrer-Gimenez & Clotet-Ballus, 1979), extracción líquido-líquido (ELL) (Callejón et al., 2008; Callejón, Morales, Troncoso, & Silva Ferreira, 2008), extracción en fase sólida (SPE) (Morales, Benitez, & Troncoso, 2004), microextracción en fase sólida (SPME) (Natera Marín, Castro Mejías, de Valme García Moreno, García Rowe, & García Barroso, 2002) y extracción por absorción con barras magnéticas agitadoras o “Stir Bar Sorptive Extraction” (SBSE) (Callejón, González, Troncoso, & Morales, 2008; Marrufo-Curtido et al., 2012) o extracción en espacio de cabeza dinámico (DHS) (Manzini et al., 2011). A pesar de la gran variedad de técnicas de muestreo que se han empleado para extraer y concentrar los compuestos volátiles de los vinagres de vino, este paso previo al análisis todavía sigue siendo un problema no bien resuelto debido a la gran variedad de vinagres de vino y las diferencias y limitaciones de las distintas técnicas de muestreo (Castro Mejías, Natera Marín, De Valme García Moreno, & García Barroso, 2002; Guerrero, Marín, Mejías, & Barroso, 2006). De hecho, no todas las técnicas de muestreo serán igualmente adecuadas para

la diferenciación y caracterización de unas muestras de vinagre u otras. Y en este contexto, aún faltan estudios sobre las diferencias que se producen en el perfil volátil obtenido por diferentes técnicas de extracción, sin haberse finalmente descrito cuál es la más adecuada para el caso del vinagre de vino.

Entre las principales limitaciones de estas técnicas encontramos que algunas de ellas requieren demasiado tiempo, implican una gran manipulación de la muestra lo que puede conducir a distintos tipos de error, así como el empleo de disolventes orgánicos (como por ejemplo en la ELL o SPE). Otras tienen baja reproducibilidad, o bien pueden producir alteraciones de los volátiles originales cuando se superan determinadas temperaturas formándose nuevos compuestos, e incluso dando lugar a una fracción de aroma determinada que no sea representativa de la muestra, como por ejemplo ocurre con la HSE (Núñez & Maarse, 1986). Por ello, los métodos que incluyan una mínima manipulación de la muestra y presenten una elevada reproducibilidad, a ser posible mediante un procedimiento automatizado, darían lugar a valores de concentración con mayor exactitud y más ajustados al aroma real de un vinagre (Guerrero et al., 2006; Marrufo-Curtido et al., 2012; Pizarro, Esteban-Díez, Sáenz-González, & González-Sáiz, 2008). Por este motivo, en los últimos años las técnicas más empleadas para la determinación del perfil volátil de vinagres de vino han sido las técnicas SPME, SBSE y DHS, por ser técnicas simples, con una alta sensibilidad y reproducibilidad, de bajo coste, automatizables, que requieren pequeños volúmenes de muestra y que generalmente no precisan del uso de disolventes orgánicos para llevar a cabo la extracción.

Las técnicas SPME y SBSE pueden aplicarse de dos maneras: mediante inmersión directa en la muestra (SPME y SBSE) o en el espacio de cabeza (HS-SPME y HSSE) (Morales, Aparicio-Ruiz, & Aparicio, 2013). Este último modo de muestreo tiene numerosas ventajas como son la reducción del riesgo de contaminación, el incremento de la vida media de la fibra o material de extracción y permite una elevada concentración del analito (Bicchi, Iori, Rubiolo, & Sandra, 2002; Weldegergis, Tredoux, & Crouch, 2007).

Tanto HSSE como HS-SPME se han aplicado en el análisis de matrices sólidas y líquidas, como por ejemplo en vinos y vinagre de vino (Callejón et al., 2008; Castro Mejías et al., 2002; Cirlini, Caligiani, Palla, & Palla, 2011; Marrufo-Curtido et al., 2012; Ubeda, Callejón, Troncoso, Peña-Neira, & Morales, 2016), mientras que DHS ha sido mucho menos aplicada en estas muestras (Manzini et al., 2011). En base a esto, DHS, HS-SPME y HSSE son las técnicas que se han empleado en este trabajo de tesis, las cuales se describen a continuación.

1.4.3.1.1.1.1. Extracción en espacio en cabeza dinámico (DHS)

La principal diferencia de la extracción en espacio de cabeza dinámico (DHS) con respecto a la extracción en espacio de cabeza estático (HS) es que arrastra los compuestos volátiles del espacio de cabeza con un flujo controlado de un gas inerte, los cuales se adhieren y concentran en un tubo relleno con un material sorbente (Figura 12). Su principal ventaja frente a otras técnicas es su alta sensibilidad y su fácil implementación. Por otro lado, los tres parámetros más importantes que afectan a esta técnica y que deben ser optimizados son la temperatura, el tiempo y el flujo de arrastre. Una mala optimización del tiempo y flujo de arrastre puede conducir a un arrastre insuficiente o inadecuado (valores bajos de flujo y/o tiempo) o a una baja recuperación de volátiles (valores altos de flujo y/o tiempo). Con respecto a la temperatura, ésta está condicionada por el tipo de compuestos que se pretenden analizar, así como por la termolabilidad de la muestra.

1.4.3.1.1.1.2. Microextraccion en fase sólida en espacio en cabeza estático (HS-SPME)

Otra de las técnicas ampliamente empleadas para analizar el perfil volátil de los vinagres de vino es la microextracción en fase sólida en espacio de cabeza estático (HS-SPME), desarrollada por Arthur y Pawliszyn (1990) (Arthur & Pawliszyn, 1990). Esta técnica se basa en la partición de los componentes orgánicos entre una muestra líquida o su fase vapor y una fina fase de revestimiento polimérico depositada en una fibra. Este recubrimiento polimérico de la fibra SPME se expone en el espacio de cabeza de un vial cerrado herméticamente hasta alcanzar el equilibrio de reparto de los compuestos volátiles entre la muestra y el recubrimiento de la fibra que constituye la trampa (Figura 12). La adsorción del analito depende tanto del equilibrio entre la matriz y el espacio de cabeza, así como del equilibrio correspondiente entre el espacio de cabeza y el recubrimiento polimérico de la fibra.

Esta técnica ofrece importantes ventajas con respecto a los métodos tradicionales de muestreo, ya que no requiere el uso de disolventes para la extracción, la extracción y concentración se llevan a cabo de manera simultánea y en un corto periodo de tiempo, y además es una técnica simple y de bajo coste (Pizarro et al., 2008). Los principales parámetros que requieren ser considerados para su optimización son el tipo de polímero de la fibra empleada, el tiempo y temperatura de extracción, la concentración de la sal y el volumen de muestra. Sin embargo, debido a la baja cantidad de polímero que es capaz de contener la fibra, su principal desventaja es una baja sensibilidad, así como la posible presencia de artefactos proporcionados

por el septum y problemas de competencia entre volátiles en su adsorción por la fibra (Baltussen, Cramers, & Sandra, 2002; Oliver-Pozo, Aparicio-Ruiz, Romero, & García-González, 2015).

1.4.3.1.1.1.3. Extracción por sorción en espacio en cabeza estático (HSSE)

Por otro lado, otra de las técnicas que se emplean en matrices como el vinagre, con similares ventajas y principios básicos que HS-SPME, es la extracción por sorción en espacio en cabeza estático (HSSE) desarrollada por Baltussen, Sandra, David y Cramers (1999) (Baltussen, Sandra, David, & Cramers, 1999). En este caso, la extracción se lleva a cabo mediante una barra magnética agitadora recubierta por un polímero de extracción, denominada comercialmente como Twister®, que se sitúa en el espacio de cabeza mediante un inserto de cristal (Figura 12). Sin embargo, esta técnica ha mostrado una mayor sensibilidad que HS-SPME debido a la mayor cantidad de polímero que contienen los Twisters® (David & Sandra, 2007).

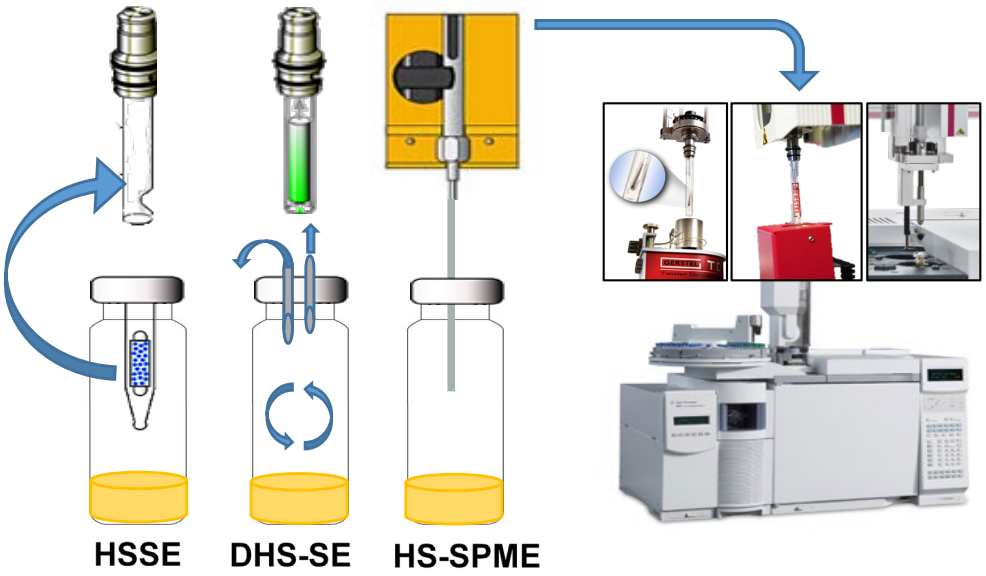


Figura 12. Esquema de las tres técnicas de extracción más utilizadas en el análisis de vinagres de vino.

1.4.3.1.1.2. Cromatografía de gases-espectrometría de masas (GC-MS)

La cromatografía de gases (GC) ha sido la técnica más utilizada para analizar la calidad del vinagre, ya que está directamente relacionada con la composición volátil del vinagre. GC es la técnica analítica oficial para la determinación del contenido de acetoína, metanol, alcoholes superiores y acetato de etilo (OENO 69-70/2000), y también se ha aplicado para determinar los polialcoholes en vinagre con la finalidad de caracterizar los vinagres de diferentes orígenes botánicos o para detectar una sospecha de adulteración de vinagres de vino con vinagre de alcohol menos costosos (Antonelli, Zeppa, Gerbi, & Carnacini, 1997). Todos estos parámetros han demostrado estar relacionados con la calidad y el origen de los vinagres de vino.

Además, la cromatografía de gases combinada con la espectrometría de masas (GC-MS), tras la previa extracción por alguno de los métodos mencionados anteriormente, ha mostrado ser la metodología más eficiente y, por tanto, más empleada para determinar la composición volátil de los vinagres. Esto es debido al alto poder de resolución de la GC y la capacidad de identificación y alta sensibilidad de la MS. Esta metodología se ha aplicado para la determinación de aldehídos volátiles como parámetros diferenciadores de los vinagres de elevada calidad (Durán-Guerrero, Chinnici, Natali, & Riponi, 2015); para la determinación del perfil volátil como parámetro de clasificación de diferentes tipos de vinagre (Chinnici et al., 2009; Cirlini et al., 2011; Cocchi et al., 2004; Pizarro et al., 2008; Ubeda et al., 2016); e incluso para la diferenciación de los vinagres de calidad con indicadores geográficos como DOP o IGP (Chinnici et al., 2009; Cocchi et al., 2004; Marrufo-Curtido et al., 2012). Respecto a esta última diferenciación, Chinnici et al. (2009) demostraron que, mediante la determinación de ácidos grasos de cadena corta, compuestos furánicos, derivados enólicos y algunos ésteres por GC-MS se consiguió discriminar tres IGP diferentes de vinagres (vinagre balsámico tradicional de Módena, vinagre balsámico de Módena y Vinagre de Jerez). De manera similar, Marrufo-Curtido et al., (2012) utilizaron GC-MS para caracterizar la composición volátil de los mismos tres IGP, mientras que Cirlini et al. (2011) la empleó para distinguir los distintos envejecimientos del vinagre balsámico de Módena (Cirlini et al., 2011).

Sin embargo, a pesar de todos los estudios aquí mencionados, los aromas de los vinagres de las DOP “Vinagre de Condado de Huelva” y de “Vinagre de Montilla-Moriles” apenas han sido estudiados. Por otro lado, aunque se han realizado diferentes estudios sobre el aroma del “Vinagre de Jerez” (Callejón et al., 2008; Callejón et al., 2008; Morales et al., 2002), es necesaria una mayor profundización sobre los compuestos activos aromáticamente y de impacto

característicos de las diferentes categorías de la DOP “Vinagres de Jerez”, así como estudiar los fenómenos de sinergia y enmascaramiento ante la presencia de otros compuestos aromáticos.

Por otro lado hay que mencionar que, a pesar del hecho de que las técnicas cromatográficas son costosas y requieren mucho tiempo de análisis, se debe tener en cuenta que en los últimos años, con el desarrollo de herramientas quimiométricas como la resolución de curvas múltiples (MCR) o el análisis factorial paralelo (PARAFAC) que se explicarán en secciones posteriores, se está abriendo una nueva vía para resolver problemas comunes en los resultados cromatográficos y para mejorar la interpretación de los datos mediante un análisis rápido y preciso (Casale, Armanino, Casolino, Oliveros, & Forina, 2006b; Cocchi, Durante, Grandi, Manzini, & Marchetti, 2008; Hantao et al., 2012).

1.4.3.1.1.3. Cromatografía de gases acoplada con olfatometría (GC-O)

El aroma es considerado como uno de los principales indicadores de la calidad de un producto. Para que una sustancia produzca sensación de olor debe alcanzar la pituitaria en cantidad suficiente para desencadenar una respuesta que sea transmitida al cerebro. Esto difícilmente se puede lograr si la sustancia no es relativamente volátil, por lo que se puede considerar que las sustancias no volátiles son inodoras y que las sustancias responsables del aroma tienen volatilidad contrastada. Por ello, el estudio del aroma se ha orientado durante muchos años al conocimiento de la composición química de sus compuestos volátiles.

En general, el aroma de cualquier materia está compuesto por uno o más compuestos volátiles que están presentes en concentraciones superiores a las de su umbral de detección olfativo en su correspondiente matriz (Delahunty, Eyres, & Dufour, 2006). Este umbral de detección se define como la concentración mínima de la sustancia capaz de ser percibida por la media de la población (Meilgaard, Civille, & Carr, 1999). Así, las sustancias que tienen un umbral de detección muy bajo pueden contribuir enormemente al aroma, incluso en concentraciones muy bajas, mientras que otras sustancias presentes en concentraciones altas pueden no contribuir al olor, al ser su umbral de detección elevado. Por tanto, para poder comprender la contribución de cualquier compuesto volátil al aroma, no basta con saber si ese compuesto está presente o ausente en la muestra, sino también conocer cómo se percibe ese compuesto a una concentración dada (Delahunty et al., 2006). En base a esto se puede distinguir entre compuestos que participan (aromas activos) y que no participan (aromas inactivos) en el aroma global de un producto, según el valor de actividad aromática (OAV) de un compuesto en una matriz determinada, que se define como la relación entre la concentración del compuesto y su

umbral de detección olfativa, expresándose en unidades de aroma (Ferreira, Pet'ka, Aznar, & Cacho, 2003). Así, se considera que una sustancia no participa en el aroma si su valor de actividad aromática es menor que la unidad, y sí que lo hace cuando dicho valor es mayor a uno, siendo la participación tanto mayor cuanto mayor es el valor de OAV (Grosch, 2001).

Sin embargo, los conceptos y datos de valores de actividad aromática deben aplicarse con precaución ya que, si bien permite juzgar de una manera objetiva la contribución de los distintos compuestos aromáticos, no tiene en cuenta ciertas limitaciones: fundamentalmente no considera los efectos sinérgicos y antagónicos de los odorantes, y no tiene en cuenta la ley psicofísica de percepción o ley de Steven (Stevens, 1971). El valor de la actividad aromática supone que la percepción es directamente proporcional a la intensidad del estímulo, y eso no es del todo correcto, ya que la intensidad percibida de un compuesto aromático irá aumentando linealmente conforme aumenta la concentración del compuesto hasta que alcanza una concentración a partir de la cual la intensidad no seguirá aumentando debido a la saturación de los receptores olfativos. Por otro lado, hay compuestos cuya intensidad percibida no aumenta proporcionalmente con el incremento de concentración, es decir, se necesitan incrementos grandes de concentración para que se perciban aumentos en la intensidad del aroma, y, por el contrario, compuestos cuya intensidad percibida aumentará significativamente con pequeños incrementos de concentración.

En este contexto, la cromatografía de gases acoplada con la olfatometría (GC-O) o "sniffing" es la técnica analítica más apropiada para determinar el impacto real de los compuestos volátiles presentes en el vinagre que contribuyen al aroma de un vinagre (aromas activos), ayudando además a caracterizar ciertos volátiles individualmente con descriptores sensoriales, así como a identificar los odorantes más relevantes conocidos también como aromas de impacto.

Esta técnica combina el análisis instrumental y sensorial simultáneamente, ya que los analitos, a medida que van eluyendo de una previa separación por cromatografía de gases, son detectados al mismo tiempo por la nariz humana y un detector convencional, como el detector iónico de llama (FID) o el detector de espectros de masas (MSD). La detección del aroma del analito es posible gracias a la presencia de un accesorio llamado puerto de olfacción que está situado al final de la columna cromatográfica tal y como se muestra en la Figura 13.

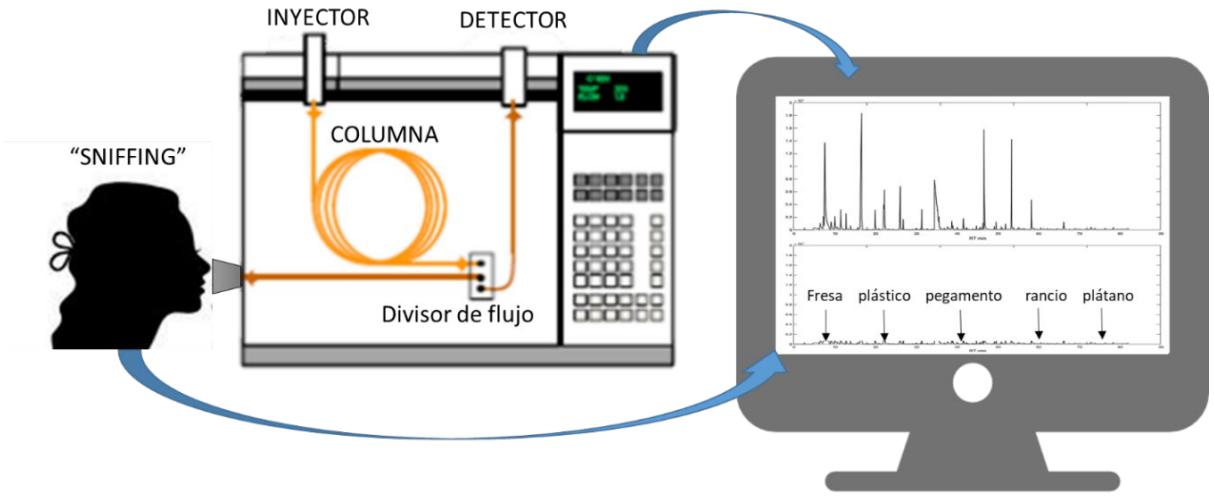


Figura 13. Esquema de un análisis por cromatografía de gases acoplado a detector olfatométrico.

Para cada compuesto que emerge del cromatógrafo de gases, el detector humano tiene la capacidad de medir la duración del olor (desde que se detecta hasta que desaparece), describir la cualidad del olor percibido y cuantificar su intensidad. Basándose en estos tres principios, se han desarrollado varias técnicas olfatométricas para determinar la importancia relativa de los odorantes de una muestra, los cuales se pueden clasificar en tres categorías: técnicas de dilución, técnicas de tiempo-intensidad y técnicas de frecuencia de impacto.

Las técnicas de dilución incluyen un proceso de dilución del extracto con un disolvente, realizándose la evaluación olfatométrica de cada dilución hasta que los odorantes de interés dejan de ser percibidos. Los panelistas que llevan a cabo el análisis (normalmente 1 ó 2 asesores) anotan cuándo detectan un olor y además dan una descripción del olor, pero no miden la intensidad aromática en ninguna de las concentraciones analizadas. Entre ellas se encuentra el análisis AEDA (acrónimo de "aroma extract dilution analysis") y el análisis CHARM ("combined hedonic aroma response measurement"), cuya principal diferencia se encuentra en la manera de registrar los datos. En general, las técnicas de dilución son capaces de discriminar diferentes muestras y además permiten considerar las modificaciones del aroma que se producen en las diferentes concentraciones, pero tienen el inconveniente de que requieren bastante tiempo para completar el análisis y por consiguiente hay más probabilidad de obtener resultados subjetivos y de menor precisión (Plutowska & Wardencki, 2008). Además, estas técnicas han sido criticadas por asumir que la intensidad aumenta proporcionalmente con la concentración en todos los compuestos aromáticos de una muestra (Pet'ka, Ferreira, & Cacho, 2005).

Por otro lado, las técnicas de tiempo-intensidad consisten en registrar la presencia o ausencia de un odorante y también la intensidad con la que se percibe, utilizándose para ello diferentes escalas de medida. En general, el principal inconveniente de las técnicas tiempo-intensidad es el intenso entrenamiento que los panelistas requieren para obtener resultados reproducibles. Sin embargo, una vez que el panel está entrenado, se pueden caracterizar los perfiles aromáticos de las muestras con una precisión mejorada (Delahunty et al., 2006).

Respecto a las técnicas de frecuencia de impacto o citación (NIF, *Nasal Impact Frecuency*), un panel formado entre 6-12 personas analizan el mismo extracto de la muestra, anotando la ausencia/presencia del odorante y describiendo su aroma. Después, se calcula el porcentaje de panelistas que son capaces de detectar un odorante en un tiempo de retención concreto (Pollien et al., 1997), considerándose que los compuestos que se detectaron con mayor frecuencia son los que tienen una mayor importancia relativa en el aroma de la muestra. Además, se asume que los resultados obtenidos están relacionados con la intensidad del olor percibido en la concentración a la que está presente el analito en el extracto (Van Ruth, 2001). Así, un odorante que ha sido detectado por todo el panel tendrá un valor de intensidad del 100 %. El beneficio fundamental de la frecuencia de impacto es su simplicidad. Además, consume menos tiempo que las otras técnicas, con muy buena reproducibilidad y los panelistas no requieren mucho entrenamiento (Plutowska & Wardencki, 2008). Por el contrario, su principal limitación está relacionada con la escala de medida, ya que un compuesto a una concentración concreta puede ser percibido por todos los panelistas, alcanzando una frecuencia máxima, pero si se incrementa su concentración, su intensidad aromática probablemente también aumentará y, sin embargo, no podrá hacerlo la frecuencia de detección (Delahunty et al., 2006). No obstante, la limitada capacidad de discriminación de esta técnica puede mejorarse si se tiene en cuenta la intensidad aromática, aunque ello requiere un entrenamiento específico del panel sensorial. Así, algunos autores emplean la llamada denominada “Frecuencia Modificada”, la cual tiene en cuenta tanto la frecuencia como la intensidad de cada odorante, y, por tanto, se puede considerar como una técnica híbrida entre ambas (Campo, Ferreira, Escudero, Marqués, & Cacho, 2006; Escudero, Campo, Fariña, Cacho, & Ferreira, 2007). La Frecuencia Modificada (FM) de cada odorante se calcula mediante la media geométrica de la frecuencia de detección de una zona aromática y la intensidad media expresada como porcentaje. Esta técnica ha demostrado proporcionar resultados más fiables que las otras debido a las capacidades discriminativas de la frecuencia de detección que se mejoran teniendo en cuenta la intensidad (Dravnieks, 1985). Es por ello que es la técnica olfatométrica que se ha empleado en este trabajo de tesis.

En general, la olfatometría ha demostrado ser una herramienta eficaz para la caracterización de aromas alimentarios y, en particular, para la caracterización del aroma de algunos vinos y vinagres (Aceña, Vera, Guasch, Busto, & Mestres, 2011; Callejón et al., 2008; Callejón et al., 2008; Charles et al., 2000). Sin embargo, a pesar de que se ha demostrado que es un método adecuado para la selección e identificación de compuestos aromáticos en matrices alimentarias, se encuentra poca investigación en la bibliografía sobre la aplicación de esta técnica en vinagres. De hecho, solo hay algunos artículos sobre la caracterización del aroma de los vinagres de vino tinto (Charles et al., 2000), de algunos vinagres chinos (Zhou et al., 2017) o de Vinagres de Jerez (Callejón et al., 2008a y b). En estos últimos estudios se determinaron los compuestos activos aromáticamente y los compuestos de impacto de los Vinagres de Jerez. Estos estudios se consideran una primera aproximación, ya que se basaron exclusivamente en el análisis de tres Vinagres de Jerez (un Vinagre de Jerez, un Reserva y un Gran Reserva) y por ello, se necesitaría y sería interesante hacer un estudio más profundo sobre esta DOP y aplicar la olfatometría a las otras dos denominaciones, Vinagre de Montilla-Moriles y Vinagre de Condado de Huelva y ver sus similitudes y diferencias.

1.4.3.2. Técnicas espectroscópicas

En los últimos años se han producido rápidos avances científicos y tecnológicos en el campo del estudio de la autenticidad de los alimentos debido al avance instrumental analítico y al mayor conocimiento sobre la composición química de los alimentos. Además, en muchos casos no es posible tomar una decisión inequívoca sobre la autenticidad de una muestra utilizando los métodos convencionales debido a la aparición de adulteraciones sofisticadas. Esto ha inducido a un perfeccionamiento en los controles analíticos. La mayoría de los métodos analíticos convencionales empleados en la caracterización y control de calidad de alimentos suelen ser costosos, destructivos y requieren mucho tiempo, además de requerir operadores calificados y tener un alto impacto ambiental. Por esta razón, las metodologías rápidas, baratas, no destructivas y directas basadas en técnicas no dirigidas son cada vez más interesantes en el enfoque de la autentificación de productos alimentarios como el vinagre de vino. Por lo tanto, la tendencia actual es buscar técnicas que permitan el análisis de un gran volumen de muestras de forma fiable y rápida, y al mismo tiempo obteniendo una amplia información sobre las características físico-químicas de la muestra. A ser posible de forma no-destructiva. Estas técnicas suelen caracterizarse por una baja selectividad de la señal, a diferencia de las técnicas dirigidas, y por ese motivo es necesaria aplicar quimiometría.

Dentro de este grupo de técnicas, existe un gran interés en la aplicación de técnicas espectroscópicas basadas en espectroscopía infrarroja (IR), fluorescencia o RMN, que permitan evaluaciones de calidad de vinagre más objetivas, rápidas y menos costosas (Versari et al., 2011). Estas técnicas permiten obtener la huella dactilar de la muestra permitiendo determinar varias propiedades simultáneamente teniendo en cuenta tanto la contribución individual como las interacciones de los diferentes componentes químicos en los vinagres (Cocchi et al., 2004). Además, otra de las ventajas de estas técnicas es que no requieren un entrenamiento altamente especializado del analista a diferencia de otras técnicas donde el entrenamiento del panelista tiene un gran impacto en los resultados.

1.4.3.2.1. Espectroscopía de infrarrojo medio (MIR)

La espectroscopía MIR se basa en la interpretación del comportamiento vibratorio de las moléculas, cuando éstas se exponen a la radiación electromagnética en el rango espectral entre 5000 y 500 cm^{-1}. Cuando la radiación infrarroja (MIR) interactúa con una molécula, esta absorbe parte de la misma a determinadas frecuencias que corresponde a grupos funcionales particulares, y el resto se transmite o refleja, por lo que los componentes bioquímicos de la

muestra determinan la cantidad y frecuencia de la luz que se absorbe, se transmite o se refleja. El espectro MIR se divide típicamente en dos regiones distintas: la región del grupo funcional (de 4000 a 1500 cm^{-1}) que incluye señales de los principales grupos funcionales (-CH, N-H, O-H) y la región de la huella dactilar (de 1500 a 500 cm^{-1}) que incluye patrones de absorción diferentes y únicos para cada compuesto.

Según la bibliografía , se ha demostrado que la espectroscopía MIR puede abordar una amplia gama de problemas y brindar soluciones para el análisis rápido de los alimentos (Bevin, Fergusson, Perry, Janik, & Cozzolino, 2006; Pillonel et al., 2003; Rodriguez-Saona & Allendorf, 2011). Esta técnica combinada con la quimiometría ha ganado una amplia aceptación para propósitos de autenticidad y clasificación en alimentos, siendo informativa a nivel molecular y produciendo una huella dactilar espectral única de cada muestra. Además, el uso de un accesorio como la reflectancia total atenuada (ATR) permite el análisis directo de líquidos de manera simple, rápida, en solo unos minutos y de manera no destructiva, lo que implica una preparación mínima de la muestra. Así, durante el análisis por ATR, cuando se hace pasar radiación IR por este cristal, ésta experimenta una reflexión total en la interfaz muestra-cristal, creándose en la muestra lo que se llama una *onda evanescente,* que penetra en ella unas pocas micras, absorbiendo la muestra parte de la radiación. Por lo tanto, es esencial que exista un contacto real de la muestra y el cristal. La cantidad de radiación que se refleja es menor que la radiación incidente, es decir, la reflexión está atenuada. El espectro que se genera de la muestra es la representación gráfica del grado en que se absorbe la radiación en función de la longitud de onda, es decir, un espectro de reflexión con bandas características de cada tipo de enlace químico presente en la muestra Figura 14.

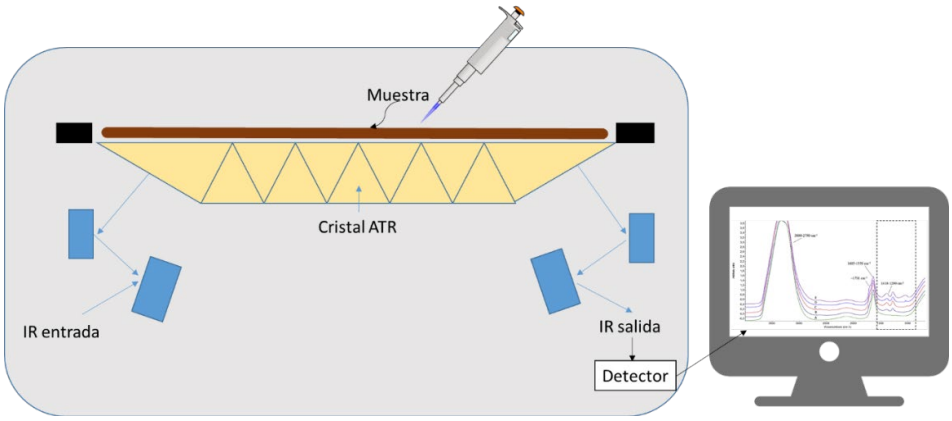


Figura 14. Esquema del análisis por ATR-FTIR de una muestra de vinagre de vino.

La metodología ATR acoplada a FTIR (espectroscopía de infrarrojo medio con transformada de Fourier) se ha estudiado para la estimación individual de compuestos de interés en los vinagres, así como para predecir la puntuación sensorial del Vinagre Balsámico Tradicional de Módena mediante el desarrollo de modelos de regresión (Versari, Parpinello, Chinnici, & Meglioli, 2011), así como para determinar el contenido en ácidos orgánicos de los vinagres en general (Regmi, Palma, & Barroso, 2012). Además, los espectros obtenidos por FTIR también se han utilizado para discriminar entre el vinagre balsámico tradicional y otros vinagres (Del Signore, 2001), así como para clasificar vinagres de diferentes materias primas y con o sin envejecimiento en madera (Guerrero et al., 2010).

En general, este método proporciona una mayor cantidad de información química en comparación con la espectroscopía de infrarrojo cercana (NIR) proporcionando ventajas en términos de asignación química de las bandas y una mayor facilidad en la interpretación de los espectros sin la necesidad de aplicar una quimiometría compleja.

1.4.3.2.2. Espectroscopía de infrarrojo cercano (NIR)

La espectroscopía de infrarrojo cercano se extiende en un rango espectral mayor que el de MIR, encontrándose en la región comprendida entre 5000 - 15000 cm^{-1} (o lo que es lo mismo de 800 a 2500 nm). Es la primera región espectral que muestra bandas de absorción relacionadas con las vibraciones moleculares y se caracteriza por las bandas armónicas y las bandas de combinación Los espectros NIR registran información sobre la absorción de radiación infrarroja de las moléculas orgánicas con enlaces heteroatómicos cuando experimentan un cambio neto en el momento dipolar como consecuencia de su movimiento de vibración o rotación, lo que implica la respuesta de los enlaces moleculares de C – H, N – H, C – O y O – H. Cuando se trata de especies homonucleares (H_2, O_2…) el momento dipolar no se altera durante la vibración o rotación, y, en consecuencia, este tipo de compuestos no absorben en el infrarrojo. Así, la composición química de la muestra origina diferencias que quedan reflejadas en las vibraciones armónicas y de combinación, construyendo de esta manera un espectro característico que sirve como huella dactilar. Esta técnica resulta de utilidad para determinar los ácidos orgánicos y el pH de una muestra.

Esta técnica, al igual que MIR, tiene las ventajas de permitir llevar a cabo los análisis con una alta velocidad, precisión, simplicidad y bajo coste, con casi ninguna preparación de la muestra, siendo además más fácilmente de implementar a escala industrial. En consecuencia, en los últimos años ha habido una creciente tendencia del uso de esta espectroscopía para el

análisis cuantitativo y cualitativo de los alimentos, y particularmente del vinagre. Así, esta técnica se ha utilizado para realizar una monitorización simultánea o "en línea" del etanol y otros compuestos relevantes para la calidad del vinagre, así como para monitorizar el proceso de producción, lo que permite evaluar acciones correctoras particulares de la manera más rápida posible. Varios trabajos de investigación también han demostrado la utilidad de la espectroscopía NIR para clasificar muestras de vinagre de acuerdo con la materia prima de origen y el proceso de elaboración. Así, Saiz-Abajo et al. (2004) utilizaron la espectroscopía NIR para diferenciar vinagres de vino y vinagres de alcohol en el norte de España, alcanzando tasas de clasificación de calibración y validación del 85.7% y 100%, respectivamente, además de demostrar la idoneidad de esta técnica para clasificar vinagres de ocho tipos diferentes con diversos métodos de procesamiento, tales como la adición de mosto, la fermentación o el envejecimiento en madera (Sáiz-Abajo, González-Sáiz, & Pizarro, 2004b). Además, se ha aplicado con éxito para la determinación de ciertos compuestos en vinagre útiles para el control de procesos de vinagre a escala industrial (Sáiz-Abajo et al., 2006), para discriminar el vinagre fermentado del vinagre mezclado (Fan et al., 2011) y para detectar vinagres adulterados (Sáiz-Abajo, González-Sáiz, & Pizarro, 2005).

Una de las desventajas de esta espectroscopía respecto a la espectroscopía MIR es que además de que los espectros NIR son más difíciles de interpretar que los MIR, y generalmente se necesita realizar un análisis multivariante de los datos para poder obtener resultados de los espectros que se generan y poder desarrollar modelos capaces de clasificar los vinagres de distintos tipos (Saiz-Abajo, Gonzalez-Saiz, & Pizarro, 2004a; Sáiz-Abajo et al., 2004b) o incluso capaces de predecir o monitorizar el proceso de envejecimiento del vinagre (Casale, Sáiz Abajo, González Sáiz, Pizarro, & Forina, 2006).

1.4.3.2.3. Espectroscopía de fluorescencia multidimensional (EFM)

La espectroscopía de fluorescencia también se ha investigado como una herramienta alternativa de control de calidad del vinagre. Aunque la espectroscopía de fluorescencia es una de las técnicas analíticas utilizadas desde hace mucho tiempo, recientemente se han desarrollado multitud de aplicaciones en tecnología de alimentos. Dentro de esta técnica, existen diferentes métodos de análisis: el convencional consiste en la medición de los espectros de excitación o emisión en una sola emisión o longitud de onda de excitación, respectivamente, mientras que un procedimiento más reciente consiste en registrar los espectros de emisión en diferentes longitudes de onda de excitación. Esta técnica se conoce como fluorescencia de emisión-excitación o fluorescencia multidimensional. De esta forma se obtiene una matriz de

Emisión-Excitación (EEM) bidimensional (Figura 15), que contiene información única de cada muestra, con la ventaja de contener más información sobre las especies fluorescentes que los espectros de excitación y emisión convencionales por separado.

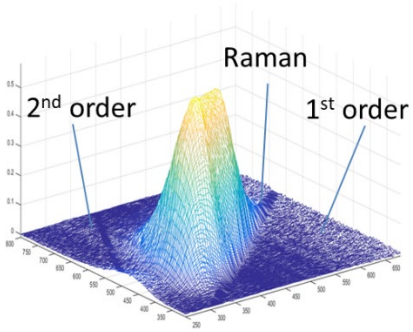


Figura 15. Ejemplo de matriz de excitación-emisión (EEM) de una muestra de vinagre de vino.

Además, el potencial de la técnica EEM puede mejorarse aplicando métodos multivariantes para el análisis de los resultados de fluorescencia, como el Análisis Paralelo de Factores ("Parallel Factor Analysis", PARAFAC) y su combinación con el análisis discriminante. PARAFAC se utiliza para descomponer los EEM de fluorescencia en diferentes componentes de fluorescencia (fluoróforos). Este método extrae la información más relevante de los datos para construir modelos más robustos de calibración y / o clasificación. Así, en un estudio reciente, Callejón et al. 2012 demostraron la capacidad de la espectroscopía de fluorescencia de excitación-emisión combinada con métodos multivariantes (Callejón, Amigo, Pairo, Garmón, et al., 2012), para caracterizar y clasificar las categorías de vinagres de vino de la DOP Vinagre de Jerez, según el tiempo de envejecimiento. Los resultados obtenidos pusieron de manifiesto el posible el uso de esta metodología como una técnica rápida para detectar muestras fraudulentas o falsificadas y asegurar la calidad del producto en comparación con otros similares del mercado. Sin embargo, este estudio se llevó a cabo analizando un número pequeño de Vinagres de Jerez, por lo que para confirmar estos resultados y crear un modelo más robusto de clasificación, que comprenda la mayor variabilidad posible de los vinagres, sería necesario analizar un mayor número de muestras. Por otro lado, esta técnica todavía no se ha aplicado para la caracterización y diferenciación de las otras DOPs existentes.

1.4.3.2.4. Espectroscopía de ultravioleta-visible (UV-vis)

Debido a los resultados satisfactorios obtenidos para el control de calidad en otros productos alimentarios (Acevedo, Jiménez, Maldonado, Domínguez, & Narváez, 2007; Azcarate, Cantarelli, Pellerano, Marchevsky, & Camiña, 2013), y en algunos vinagres (Xie, Bu, Peng, & Li, 2011), la espectroscopía ultravioleta es otra técnica que podría ser estudiada para la discriminación y clasificación del vinagre de vino.

La espectroscopía UV-vis es una técnica basada en la medición de la absorción, por las moléculas, de radiación electromagnética (luz) de las regiones ultravioleta y visible, comprendiendo las regiones del espectro de 190 nm a 800 nm de longitud de onda (Skoog, Holler, & Crouch, 2016). La posición espectral de una banda de absorción es indicativa de la presencia o ausencia de ciertas características estructurales de compuestos. Las principales razones del interés por esta metodología son su amplia aplicabilidad, la rapidez del análisis, la ausencia de residuos generados y su facilidad de uso sin costes ni operadores calificados. Además, también tiene una alta sensibilidad, selectividad moderada-alta y buena precisión (Esslinger, Riedl, & Fauhl-Hassek, 2014). Por lo tanto, algunos autores han estudiado la posibilidad de discriminar vinos de distintas denominaciones mediante el uso de espectroscopía UV-vis y procedimientos quimiométricos como el análisis de componentes principales (PCA) y el modelado independiente suave por analogía de clase (SIMCA) (Azcarate et al., 2013; Urbano Cuadrado, Luque De Castro, & Gómez-Nieto, 2005; Urbano, Luque de Castro, Pérez, Garcíaa-Olmo, & Gómez-Nieto, 2006). Todas estas características la convierten en una técnica adecuada para controlar los procesos de producción, así como para monitorizar y evaluar la composición y calidad de los productos alimentarios y bebidas, así como muestras farmacéuticas y biológicas (Wang et al. 2008; Van Den Broeke, Langergraber, y Weingartner 2006). Además, el desarrollo de dispositivos portátiles de espectroscopía UV-vis está siendo investigado con el fin de autentificar alimentos y bebidas, aumentando todo ello el interés en esta espectroscopía.

1.4.3.2.5. Espectroscopía de resonancia magnética nuclear (RMN)

La espectroscopía de RMN tiene la ventaja de ser una técnica analítica no selectiva, que recientemente ha logrado una aceptación general como una herramienta poderosa para la determinación de la calidad y autenticidad del vinagre, siendo principalmente aplicado hasta la fecha a vinagres balsámicos de Módena y sus IGP así como a algunos vinagres brasileños (Boffo, Tavares, Ferreira, & Ferreira, 2009; Consonni et al., 2008a; Consonni & Gatti, 2004; Graziosi et al., 2017). Ésta técnica permite obtener información sobre la composición química y permite la

determinación rápida y simultánea de los metabolitos hidrosolubles del vinagre, como azúcares, ácidos y flavonoides. Además tiene la capacidad de proporcionar información estructural y cuantitativa sobre una amplia gama de especies químicas en un solo análisis (Fotakis et al., 2013). Esto hace que esta técnica sea otra herramienta útil para la obtención de huellas dactilares de muestras pudiéndose aplicar en la autenticidad de los alimentos y el control de calidad. Por otro lado, también ofrece una notable selectividad y permite la identificación de compuestos desconocidos con alta reproducibilidad y repetibilidad. La técnica de RMN más comúnmente aplicada para la autentificación de origen, y recientemente reconocida como un método oficial, es la SNIF-RMN de deuterio (fraccionamiento isotópico natural específico del sitio estudiado por espectrometría de resonancia magnética nuclear).

Además, la resonancia magnética nuclear de protones (^{1}H-RMN) ha sido ampliamente utilizada para la caracterización y autentificación de ciertos vinagres. Así, esta técnica ha permitido la determinación rápida de compuestos tales como carbohidratos, ácidos orgánicos, alcoholes, polioles y sustancias volátiles relevantes para la discriminación de vinagres balsámicos (Caligiani, Acquotti, Palla, & Bocchi, 2007). Por otra parte, Papotti et al. (2015) utilizaron espectros de ^{1}H-RMN, resonancia magnética nuclear de carbono 13 (^{13}C-RMN) y espectros de coherencia cuántica única heteronuclear ^{1}H-^{13}C (HSQC), junto con análisis de datos estadísticos multivariante, en la caracterización del vinagre balsámico de Módena y vinagre balsámico tradicional de Módena (Papotti et al., 2015). Consonni et al., (2008b) también estudiaron el poder de la espectroscopía ^{1}H-RMN en combinación con la quimiometría para caracterizar y discriminar el vinagre balsámico y el vinagre balsámico tradicional de Módena, así como la aplicabilidad de la espectroscopía ^{13}C-RMN para determinar las prácticas fraudulentas presentes en las muestras (Consonni et al., 2008b; Consonni et al., 2008). Boffo et al., (2009) demostraron el potencial de ésta misma técnica en la discriminación de vinagres brasileños de acuerdo con sus materias primas como el vino, la manzana y el vinagre de alcohol (Boffo et al., 2009). Finalmente, se ha estudiado recientemente un enfoque novedoso de RMN bidimensional para la clasificación de vinagres balsámicos de Módena (Graziosi et al., 2017). Sin embargo, debido a la gran cantidad de datos que genera esta técnica, se requieren nuevamente los métodos multivariantes de tratamiento de datos, es decir, se requiere la combinación de esta técnica con la quimiometría para alcanzar los objetivos de clasificación y caracterización completa de las muestras de vinagre.

1.5. QUIMIOMETRÍA

Como se ha mencionado anteriormente, se podrían emplear muchas técnicas cromatográficas y espectroscópicas para conseguir caracterizar, clasificar o autentificar los vinagres de vino. El problema es que, a pesar de las ventajas que caracterizan a todas estas técnicas anteriormente descritas, se debe tener en cuenta que debido a que los datos espectrales lo forman miles de variables, es casi imposible interpretarlos sin la ayuda de la quimiometría, y hoy en día no se entiende una técnica sin la otra (Lohumi, Lee, Lee, & Cho, 2015). De hecho, los datos que se obtienen están formados por un numero alto de variables que definen una muestra, lo que implica que el analítico tiene que lidiar muchas veces con una gran cantidad de números. Esta es la razón básica por la cual, en el problema de la autentificación de alimentos, se utiliza cada vez más la quimiometría. Con ayuda de la quimiometría se podría afrontar con mayor probabilidad de éxito, las dificultades que surgen al emplear dichas técnicas para el control de calidad de los vinagres de vino, como serían las señales no resueltas, la gran cantidad de componentes que forma una muestra de vinagre y la complejidad de los espectros de infrarrojo (Caballero, Ríos-Reina, & Amigo, 2019).

La quimiometría es una disciplina analítica que utiliza métodos estadísticos y matemáticos para lograr una evaluación objetiva de los datos mediante la extracción de la información más importante de colecciones de datos químicos (Massart, Vandeginste, B. G. M. Buydens, de Jong, Lewi, & Smeyeres-Verbeke, 1997). O de forma más resumida, la quimiometría es un campo interdisciplinario que involucra el análisis multivariante, la matemática, la informática y la química analítica que sirve para extraer información de manera eficiente utilizando el análisis de datos multivariante. La ventaja de acoplar el análisis de datos multivariante y las poderosas herramientas para la representación e interpretación de datos, con el análisis químico, es extremadamente relevante ya que permite la construcción de modelos cuantitativos para evaluar la autenticidad y la calidad de un alimento, predecir el contenido de los constituyentes e incluso clasificar muestras desconocidas. En particular, para problemas como la autentificación de la calidad, la verificación del cumplimiento del etiquetado o la trazabilidad del origen, es importante verificar si una muestra pertenece o no a una clase específica, y todos estos problemas en términos estadísticos se encuentran en el ámbito del reconocimiento de patrones.

La quimiometría (o análisis de datos multivariante) ha demostrado tener muchas aplicaciones en la determinación cuantitativa y cualitativa de parámetros químicos para evaluar la autenticidad de productos alimentarios (Yu, Low, & Zhou, 2018), proporcionando resultados

poderosos en enfoques dirigidos y no dirigidos para identificar diversas situaciones de fraude alimentario o para certificar su origen geográfico o biológico (Beale, Morrison, Karpe, & Dunn, 2017; Martínez Bueno, Díaz-Galiano, Rajski, Cutillas, & Fernández-Alba, 2018). Otra ventaja del uso de la quimiometría es la capacidad de obtener un perfil completo o una huella dactilar de una muestra analizada mediante algunas de las técnicas mencionadas anteriormente. Por estas razones, hoy en día la mayoría de los estudios de caracterización de productos alimentarios a través de técnicas espectroscópicas utiliza herramientas quimiométricas, obteniéndose resultados exitosos (Consonni et al., 2008a; Duarte, Barros, Almeida, Spraul, & Gil, 2004; Erich et al., 2015; Karoui & De Baerdemaeker, 2007; Mazerolles, Devaux, Dufour, Qannari, & Courcoux, 2002). Pero además las herramientas quimiométricas se utilizan hoy en día incluso con técnicas cromatográficas, permitiendo corregir errores muy comunes generados por estas técnicas en los resultados finales e incluso acortar y simplificar la obtención de sus resultados (Hantao et al., 2012).

Los métodos quimiométricos se pueden agrupar de muchas maneras diferentes. El más directo es atendiendo a su principal objetivo: reconocimiento de patrones, resolución, regresión (calibración) y clasificación. La Figura 16 resume los métodos más populares dentro de cada grupo (Caballero et al., 2019). Como muestra esta figura, son muchas las técnicas quimiométricas que se pueden utilizar, pero en esta sección se describirán aquellos métodos quimiométricos utilizados en cada uno de los capítulos de la presente tesis doctoral.

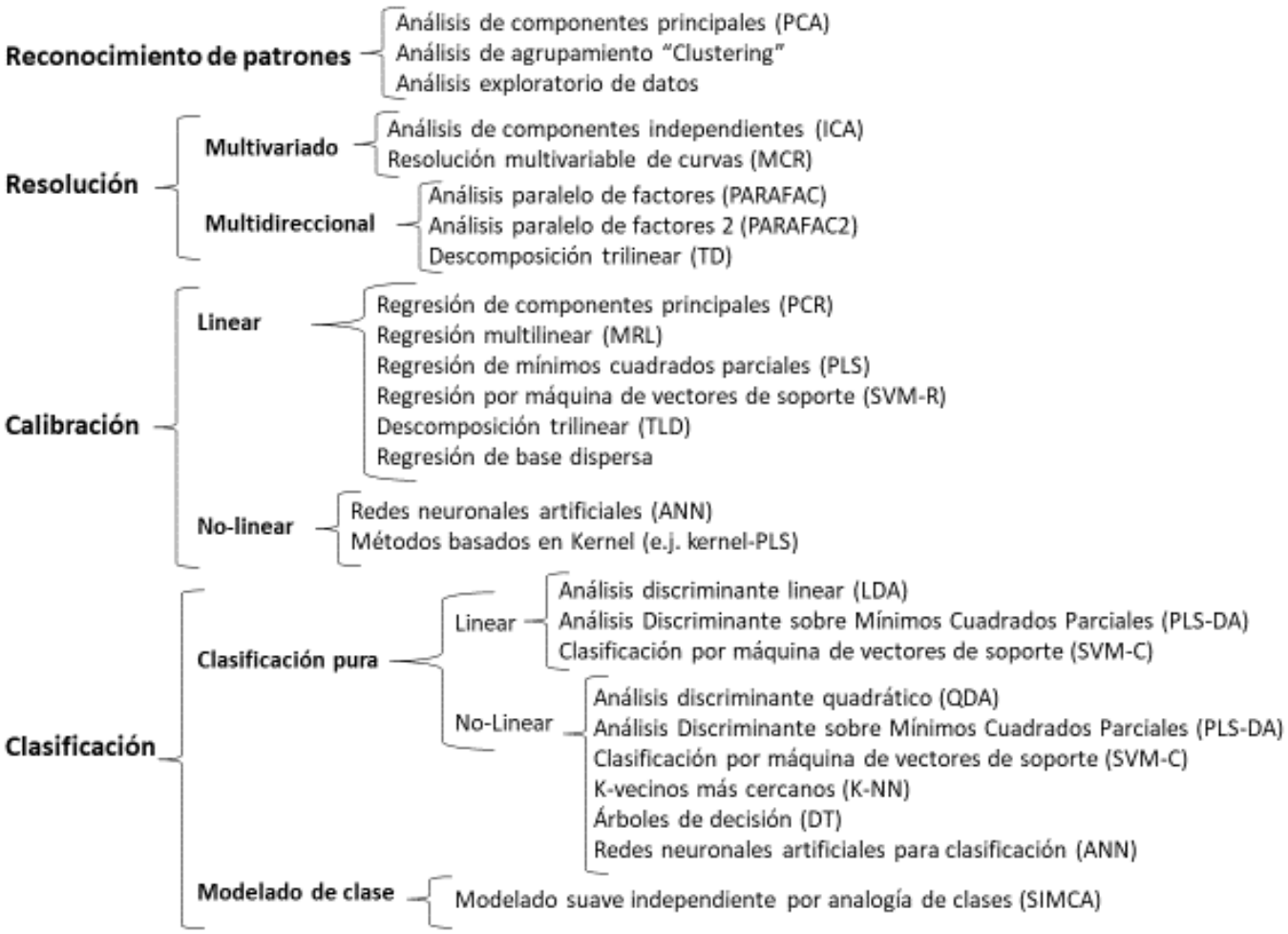


Figura 16. Esquema de los principales métodos quimiométricos aplicados en el contexto de la autentificación, caracterización y clasificación en los vinagres de vino. Capítulo de libro Caballero, Ríos-Reina & Amigo. *Comprehensive Foodomics*, 2019 **(ANEXO III**).

1.5.1. PRE-PROCESADO Y NORMALIZACIÓN DE LOS DATOS

De manera general, el pre-procesado es la modificación previa de los datos que es necesario realizar antes de construir un modelo matemático o antes de aplicar cualquier análisis de los datos. Es decir, después de construir y organizar adecuadamente la matriz de datos analíticos, el siguiente paso necesario es realizar el pre-procesamiento de los datos, siendo éste un paso crucial para obtener buenos resultados en el análisis multivariante (Bro, 1998). El uso del pre-procesado previo a la construcción de cualquier modelo ayuda a mejorar la calidad de los mismos, elimina información no útil, ruidos o línea base, resaltando la información verdaderamente útil y permite normalizar las variables que tengan distinta escala antes del análisis estadístico o multivariante. Un ejemplo de esto es el caso de los datos obtenidos por espectroscopía vibracional, los cuales necesitan ser pre-procesados casi siempre debido a que se ven afectados por numerosas fuentes de variabilidad indeseadas como efectos de dispersión de la luz, *scattering* o ruido instrumental. Pero también hay que tener especial cuidado en el pre-procesado que se elige, ya que una mala elección o un sobreprocesado puede arruinarte el modelo final.

Existen diferentes metodologías de pre-procesamiento, y la selección del procedimiento de pre-procesamiento adecuado depende principalmente de la naturaleza de los datos. Aquí, además de los más conocidos como la media y la desviación estándar que permiten evaluar las características de las poblaciones de datos, se van a citar aquellos pre-procesados que se utilizarán en los bloques que conforman el presente proyecto de tesis.

1.5.1.1. Variable aleatoria normal tipificada (SNV, *Standard Normal Variate*)

SNV es una transformación que se aplica habitualmente a los datos espectroscópicos NIR para minimizar los efectos de la dispersión de la luz, es decir, ayuda a corregir las variaciones de la línea base en los distintos espectros. Esta herramienta estandariza cada espectro manipulando únicamente los datos del mismo, mediante el centrado y escalado de cada espectro individual. En la práctica, SNV se utiliza con el fin de minimizar las interferencias multiplicativas de la dispersión en los datos espectrales producidas por los distintos tamaños de partícula en la muestra (Barnes, Dhanoa, & Lister, 1989).

La ecuación por la que se realiza este pre-procesado es la siguiente:

$$\mathbf{x}_{ik} = (\mathbf{x}_{ik} - \mathbf{m}_i)/\mathbf{s}_i$$

donde x_{ik} es la medida espectral a una longitud de onda para la muestra ***i***, m_i es la media del espectro ***k*** para la muestra ***i***, y s_i es la desviación estándar de la misma medida ***k***.

1.5.1.2. Suavizado (SMT, *Smoothing*)

SMT es otra herramienta principalmente utilizada en datos espectroscópicos. para eliminar el ruido del espectro, y como bien dice su nombre, suavizar el espectro. El suavizado asume que las variables que están cercanas entre sí en la matriz de datos (es decir, columnas adyacentes) están relacionadas entre sí y contienen información similar que puede promediarse para reducir el ruido sin pérdida significativa de la señal de interés. El algoritmo requiere la selección tanto del tamaño de la ventana (ancho del filtro) como del orden del polinomio. Cuanto más grande sea la ventana y menor el orden del polinomio, más suavizado se produce.

1.5.1.3. Corrección de la línea base (*baseline correction*)

Existen diferentes maneras de hacer la corrección de la línea base; en la presente memoria se ha utilizado el método de mínimos cuadrados ponderados (WLS, Weighted Least Squares). Este método es comúnmente empleado en aplicaciones espectroscópicas o cromatográficas donde la señal de algunas variables es debida solamente a la señal de fondo. Estas variables sirven como referencia para determinar cuanta señal de fondo debe ser eliminada de las variables cercanas. El algoritmo WLS emplea un enfoque automático para determinar qué puntos son los más probables para ser sólo línea base. Se asume que los puntos debajo de la línea base son más significativos en el ajuste de la señal de fondo del espectro. El efecto práctico de su uso es una eliminación automática de la señal de fondo, evitando la creación de picos intensamente negativos (Daszykowski & Walczak, 2006).

1.5.1.4. Centrado en la media (MC, *mean centering*)

Éste pre-procesado o normalización calcula el valor medio de cada columna de la matriz de datos para posteriormente restar este valor de la columna, trasladando los ejes del sistema de coordenadas hacia el centroide de los datos haciendo que cada muestra exhiba solo las diferencias que tiene con respecto a la muestra promedio de los datos originales (Brereton, 2007). Es decir, para cada variable se le substrae la media de esa variable. Este método es uno de los métodos de pre-procesamiento más comunes, tanto en datos discretos o variables

independientes (concentraciones, áreas, etc), como en datos continuos o variables dependientes (espectros, perfiles, etc). Otra forma de interpretar los datos centrados en la media es que, después del centrado medio, cada fila de los datos centrados en la media incluye solo cómo esa fila difiere de la muestra promedio en la matriz de datos original. En la Figura 17 se muestra un esquema de su procedimiento.

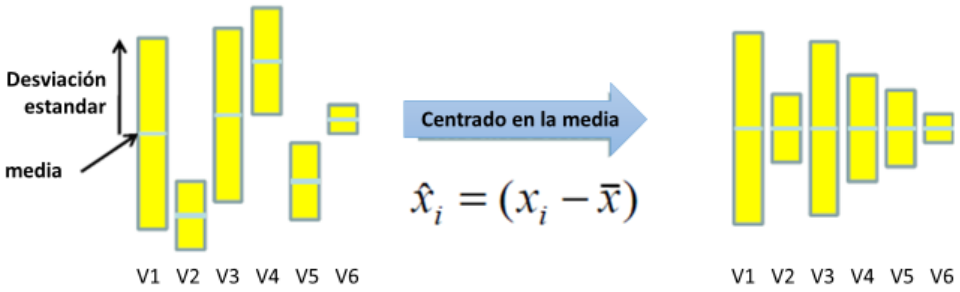


Figura 17. Esquema del pre-procesado por centrado en la media o mean center.

1.5.1.5. Escalado y autoescalado (scaling and autoscaling)

El escalado es otro de los métodos de pre-procesado de datos comúnmente utilizado con técnicas de análisis multivariante. Muchas técnicas asumen que la magnitud de una medición es proporcional a su importancia y que el nivel de ruido es similar en todas las variables. Cuando las variables tienen escalas significativamente diferentes simplemente porque están en unidades diferentes, la magnitud de los valores no es necesariamente proporcional al contenido de la información. Del mismo modo, la escala también es un problema cuando algunas variables contienen más ruido que otras variables. El escalado de las variables ayuda a resolver estos problemas escalando cada variable (columna) de una matriz de datos por algún valor, dando al contenido de información de esa variable el mismo valor que el de las otras variables. Este método es útil para evitar la presencia de valores extremos en la escala de los datos en algunas muestras y se prefiere su uso cuando se trata de aplicaciones cuantitativas, es decir, en datos discretos (Fan, Cheng, Ye, Lin, & Qian, 2006).

Por otro lado, el autoescalado une el centrado en la media con el escalado Figura 18. Este es un método de pre-procesamiento más común que utiliza la mediación centrada seguida de la división de cada columna (variable) por la desviación estándar de esa columna. El resultado es que cada columna tiene un promedio de cero y una desviación estándar de uno.

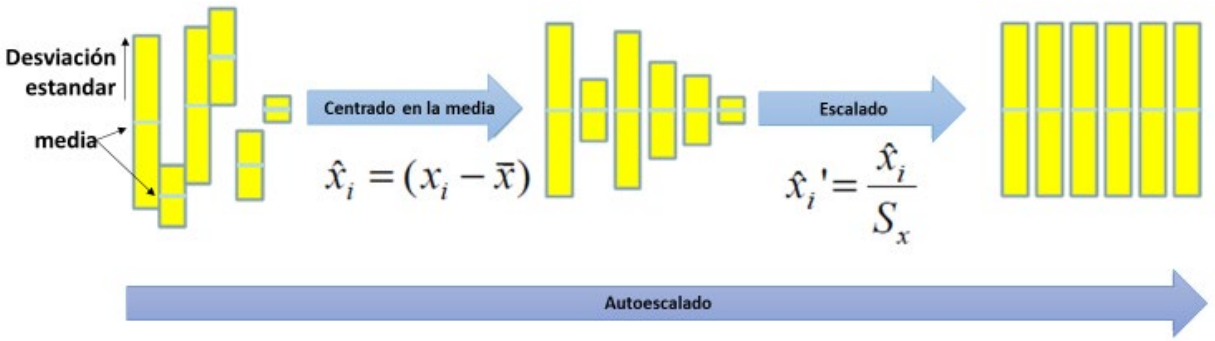


Figura 18. Esquema del pre-procesado por Autoescalado o Atuoscaling.

Este enfoque es válido para corregir diferentes escalas de las variables o diferentes unidades, siempre y cuando la fuente predominante de dicha varianza sea la señal en lugar del ruido. En estas condiciones, cada variable se escalará de tal forma que su señal útil tenga el mismo nivel que la señal de las otras variables. Sin embargo, si una variable tiene contribuciones de ruido significativas, es decir, una baja relación señal/ruido, entonces el escalado automático hará que el ruido de esta variable sea igual a la señal de otras variables, influyendo esto negativamente en el modelo. En este caso, se recomienda excluir esas variables o no aplicar este pre-procesado.

1.5.2. MÉTODOS NO SUPERVISADOS, EXPLORATORIOS O DE RECONOCIMIENTO DE PATRONES

El reconocimiento de patrones es, entre los cuatro grupos indicados en la Figura 16, los únicos que se pueden clasificar puramente como no supervisados. Es decir, no necesitan un paso previo de calibración para encontrar patrones ocultos en los datos. El propósito de los métodos no supervisados es identificar grupos o relaciones entre muestras, sin ningún conocimiento previo de clases o grupos. Se utilizan para estudiar si una muestra desconocida es similar o no a un conjunto de muestras auténticas, además de para comprimir o reducir el número de variables.

1.5.2.1. Análisis de componentes principales (PCA)

Entre los métodos no supervisados, el método más utilizado en cualquier tipo de datos es el análisis de componentes principales (PCA). El PCA es útil para dilucidar la naturaleza compleja de las relaciones multivariadas utilizando técnicas de mapeo y visualización para comprender la estructura de conjuntos de datos complejos multivariados (Bro et al., 2002; Elmqvist & Fekete, 2010). Es decir, es una técnica exploratoria comúnmente utilizada para

revelar patrones ocultos en datos complejos, que describe la variación en datos multivariantes mediante la combinación lineal de un conjunto de variables, posiblemente correlacionadas entre sí, reduciéndolas a un número pequeño de variables no correlacionadas llamadas Componentes principales "PCs". Por ello, el PCA es un método muy útil para la compresión de datos. Los PCs son, por tanto, combinaciones lineales de variables originales definidos por vectores ortogonales entre sí (Joliffe, 2002; Wold, 1987).

La principal característica de un PCA es que, combinando las variables originales en un número de nuevas variables, se puede mantener y extraer la máxima cantidad de información presente en los datos originales. Además, debido a que el objetivo principal del PCA es capturar la máxima información convirtiendo la matriz original en una lo más simple posible, intentando que el número de PCs seleccionados sea el menor posible. No todos los componentes principales contienen la misma información; los primeros son los que describen la mayor variabilidad de los datos, que se asocia a la información más relevante, mientras que los últimos describen variaciones en los datos que pueden ser debidas a ruido o error experimental, o a un sobreajuste del modelo y pueden ser descartados, con lo que se consigue una importante reducción del número de variables, así como de ruido y de información redundante. Y esto ofrece otra ventaja que puede explicarse desde un punto de vista geométrico: suponiendo que la matriz de datos original tiene *M* objetos y *N* variables, proyectar todas estas variables en el espacio sería casi imposible. Sin embargo, cuando se calculan los PCs, los *N* objetos pueden ser entonces proyectados en el espacio de dimensiones iguales al número de PCs, siendo el número de dimensiones menor al número de variables, principalmente debido a que la mayor parte de la varianza de los datos es está contemplada en los primeros PCs. Por tanto, otra de las ventajas que ofrece PCA es que, desde un punto de vista exploratorio, los datos pueden ser visualizados en un espacio de dos o tres dimensiones, llamados "*score plots*".

El procedimiento matemático de PCA se basa en la siguiente ecuación:

$$\mathbf{X}_{mn} = \sum_{i=a} \mathbf{t}_i \mathbf{p}_i^T + \mathbf{E}$$

siendo **X** la matriz de datos a tratar, de dimensiones *m* x *n*, dividida por PCA en dos matrices: ***t***, que es la matriz de puntos de coordenadas en el espacio del PCA que contiene las nuevas variables o también llamados scores o; y ***p***, la matriz de *loadings*, que contiene el peso de contribución de cada variable original sobre la combinación lineal con el superíndice *T* que se refiere a la matriz transpuesta. **E** es una matriz de error, es decir la variación residual de **X** que no es explicada por el modelo con **a** componentes principales.

1.5.3. MÉTODOS SEMI-SUPERVISADOS, MÉTODOS DE DESCOMPOSICIÓN DE MÚLTIPLES VÍAS, O MÉTODOS DE RESOLUCIÓN MÚLTIPLE (*N-WAY METHODS*)

Muchos de los datos obtenidos del análisis de alimentos proceden de diferentes o múltiples métodos y por tanto van a proporcional, diferentes informaciones o variables de distinta naturaleza (como por ejemplo GC-MS, fluorescencia, etc.). En estos casos existen métodos de descomposición de matrices de datos múltiples (N-way matrix) como son el análisis paralelo de factores (PARAllel FACtor Analysis, PARAFAC) o la resolución de múltiples curvas (Multple curve resolution, MCR) los cuales van a ser utilizados en el presente proyecto de tesis.

1.5.3.1. Análisis paralelo de factores (PARAFAC)

PARAFAC es un método de descomposición de matrices dimensiones, y más concretamente, de matrices con estructura trilinear o matrices de tres dimensiones (3D). Básicamente es un método de resolución de curvas que descompone una matriz tridimensional en tres sub-matrices bidimensionales, PARAFAC como se muestra en la Figura 19.

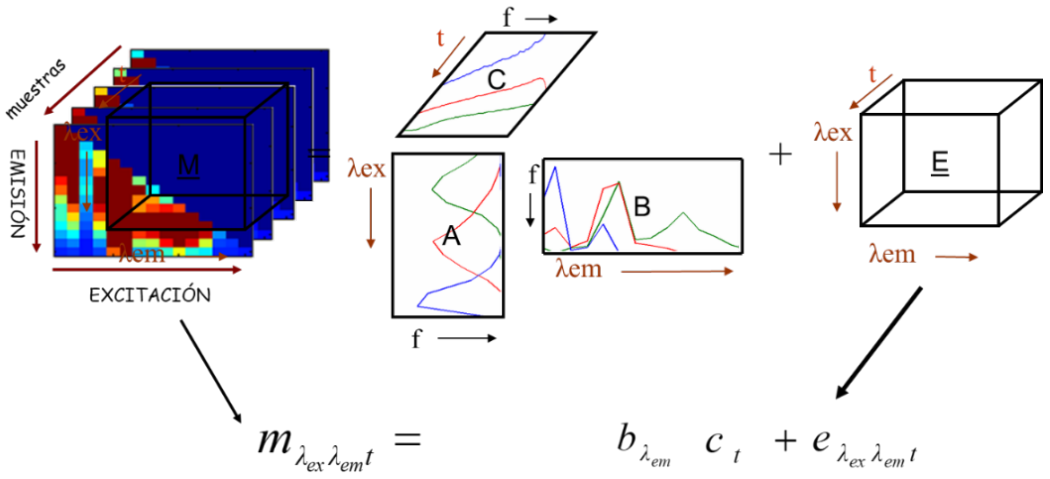


Figura 19. Representación esquemática de la descomposición por PARAFAC de una matriz de excitación-emisión de fluorescencia multidimensional.

La ecuación en la que se basa PARAFAC es la siguiente:

$$\underline{\mathbf{X}}_{ijk} = \sum_{f=1}^{F} \mathbf{a}_{if}\,\mathbf{b}_{if}\mathbf{c}_{kf} + \mathbf{e}_{ijk}$$

siendo **X** un cubo de dimensiones *I* x *J* x *K* que es descompuesta en tres nuevas matrices: una primera matriz **A** que contiene los *scores* ($\boldsymbol{a}_{if}$), y dos matrices **B** y **C** que contiene los *loadings* ($\mathbf{b}_{if}$)

y ($\mathbf{c}_{kf}$), junto con una matriz **E** ($\mathbf{e}_{ijk}$) que contiene los residuales con la información no explicada por las anteriores.

Los factores o componentes que se extraen en este caso no son forzados a ser ortogonales, como ocurría con los PCs en PCA, dando PARAFAC una solución única, siempre y cuando a diferencia también de PCA. Así, una de las principales ventajas de PARAFAC con respecto a otras técnicas de resolución es la singularidad de la solución. Es decir, no hay ambigüedades matemáticas en el modelo final, siempre y cuando se elijan bien el número de componentes a extraer y se fijen ciertos parámetros. Sin embargo, aunque parezca contradictorio, la selección del correcto número de componentes en PARAFAC no es algo fácil. Para ello, lo más utilizado es la aplicación del conocimiento previo que se tenga de la muestra, el estudio de los residuales y el estudio de tres parámetros: la consistencia central o del núcleo, llamada *core-consistency*, que mide la robustez de la descomposición matemática, cuanto más cercana a 100% mejor; así como el menor número de iteraciones y la similitud entre múltiples modelos PARAFAC. Además se están desarrollando nuevos métodos para la selección de los componentes, como el llamado CORCONDIA (Bro & Kiers, 2003). Si se ajustan estos parámetros correctamente, el modelo PARAFAC permite proporcionar una descripción química completa de las moléculas involucradas en muestras de composición compleja, lo cual ha demostrado ser útil para la caracterización de muestras de vinagre de alta calidad por técnicas como la espectrometría o la fluorescencia multidimensional (Callejón, Amigo, Pairo, Garmán, et al., 2012; Cocchi, Durante, Marchetti, Armanino, & Casale, 2007).

1.5.3.2. Resolución multivariante de curvas (MCR)

Los métodos de resolución de curvas son un conjunto de técnicas cuyo objetivo es describir la contribución subyacente correcta de un conjunto de datos. Estos métodos apuntan a resolver mezclas, dado el número correcto de constituyentes, sus perfiles de respuesta (por ejemplo, espectral, tiempo o perfiles de elución) y su influencia relativa de la señal en la muestra (Amigo, Skov, & Bro, 2010). La resolución Multivariante de Curvas-Mínimos Cuadrados Alternos (Multivariate Curve Resolution-Alternating Least Square, MCR-ALS) es quizás la metodología de resolución de curvas que mayor aplicabilidad ha demostrado a la hora de resolver problemas diversos en matrices alimentarias (Azzouz & Tauler, 2008; Hantao et al., 2012; Lachenmeier & Kessler, 2008).

En la mayoría de las situaciones, los métodos de resolución de curvas necesitan cierta información a priori (número de componentes, estimaciones iniciales sensibles, información de

selectividad) para minimizar el gran problema de las ambigüedades que tienen estas metodologías. Así, los pasos fundamentales para la aplicación de MCR con el fin de obtener una resolución última de este método son similares a los de PARAFAC: i) número de componentes; ii) estimaciones iniciales; iii) restricciones. En el caso del uso de mínimos cuadrados alternos, para determinar la solución MCR se necesita una estimación inicial de la concentración o de la matriz, por lo que la cantidad de componentes tiene que ser ajustados previamente.

Matemáticamente, MCR se puede expresar con la siguiente ecuación:

$$\mathbf{m}_{jk} = \sum_{r=1}^{R} \mathbf{C}_{jr}\mathbf{S}_{kr}^{T} + e_{jk}$$

Así, la matriz **m** de dimensiones *J* x *K*, se descompone principalmente en dos sub-matrices: **C** y **S**. Pero esta ecuación puede definirse de manera global de la siguiente forma:

$$\mathbf{M=CS^{T}+E}$$

dónde **C** y **S** son las sub-matrices en las que se divide la matriz **M**, junto con **E** que es la matriz de los residuales. Hay de nuevo algunos aspectos en común con PCA, como que ambos son métodos de descomposición de matrices que describen los datos originales como un producto de dos sub-matrices que resumen la información relevante de las muestras y la contribución de las variables. Además, ambos métodos dan soluciones que no son únicas, a diferencia de PARAFAC, utilizando PCA la ortogonalidad para resolver esta ambigüedad rotacional mientras que MCR utiliza otras restricciones que se explican más adelante. Otra diferencia entre ambos métodos son por ejemplo que los componentes de MCR no son ortogonales ni secuenciales.

En concreto, en el caso de datos de cromatografía de gases-espectrometría de masas (GC-MS), en los que se aplicará dicho método en la presente tesis, los datos en bruto se descomponen en un conjunto de perfiles de elución (concentración) y en los espectros de masas de componentes puros. Sin embargo, como ya hemos dicho, ciertas condiciones deben cumplirse para realizar una MCR adecuada (Hantao et al., 2012). Así, la presencia de ambigüedades de rotación y de soluciones no únicas en los resultados de MCR disminuye su fiabilidad y dificulta su evaluación. Se han propuesto restricciones como la no-negatividad (que fuerza a las concentraciones a ser positivos), la unimodalidad (que fuerza al perfil a tener un máximo único), el rango local y la selectividad (relacionada con la información presente/ausente de los componentes en el experimento), para limitar las ambigüedades de rotación e intensidad utilizando métodos MCR. Las restricciones de no negatividad y unimodalidad están vinculadas a

las propiedades químicas del sistema, mientras que la selectividad es una restricción matemática, relacionada con los sub-espacios del set de datos múltiple.

Además, en el caso de trabajar con un set de datos constituido por señales muy complejas, como por ejemplo una muestra formada por numerosos compuestos que generan un gran número de picos en el cromatograma, se prefiere trabajar mediante la división del espectro o cromatograma completo en secciones, lo que agiliza el cálculo ya que facilita la selección del número de componentes, así como proporciona resultados más exactos y la cuidadosa aplicación de las restricciones. La selección de estas pequeñas secciones o ventanas se suele realizar mediante inspección visual. En esta tesis, este será el procedimiento elegido para trabajar.

Pero a pesar de estas desventajas, los métodos de resolución de curvas se han utilizado ampliamente en cromatografía, donde han demostrado su versatilidad para resolver problemas cromatográficos comunes y resolver picos que de otra manera son difíciles de resolver. Un ejemplo de esto se muestra en la Figura 20, donde se muestra la versatilidad del MCR para resolver diferentes problemas cromatográficos comunes, como son la línea base o la superposición de picos cromatográficos. Así, como se muestra en esta figura, lo que a simple vista parecía un pico cromatográfico relacionado con un solo compuesto, tras aplicarle MCR, resultan ser dos picos cromatográficos que estaban solapados relacionados con la presencia de dos compuestos diferentes (picos en verde y rojo de la Figura 20), además de un tercer componente extraído que es la línea base. Por lo tanto, con este método se consigue solucionar en este caso, el problema de la línea base, así como el de la superposición o solapamiento de picos cromatográficos, lo cual nos permite obtener mejores y más completos resultados.

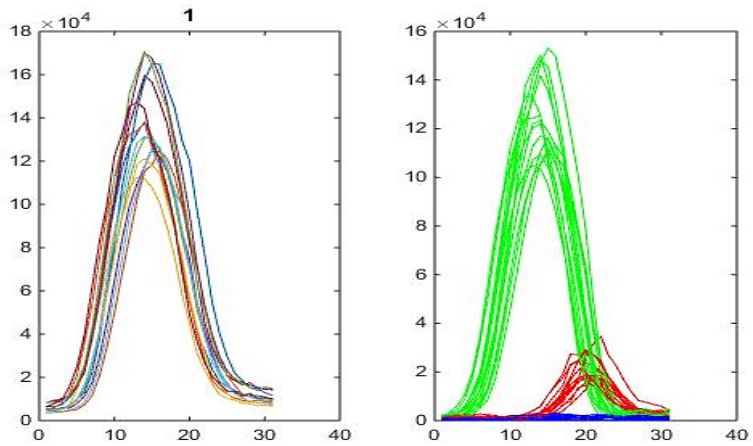


Figura 20. Ejemplo de resolución de un pico cromatográfico mediante la aplicación de MCR.

1.5.4. MÉTODOS SUPERVISADOS

1.5.4.1. Métodos de regresión o calibración linear

En esta sección se mencionarán las técnicas multivariantes empleadas en esta memoria de tesis para buscar una relación entre la señal analítica y alguna propiedad de la muestra.

1.5.4.1.1. Regresión de mínimos cuadrados parciales (PLS)

La regresión de mínimos cuadrados parciales (PLS) es un método que se utiliza para encontrar las relaciones fundamentales entre las variables independientes (**X**) y las variables dependientes (**Y**), las cuales son modeladas simultáneamente teniendo en cuenta no sólo la varianza de **X**, sino la covarianza entre **X** e **Y**. Uno de los algoritmos más usados para este fin es el denominado NIPALS (*Non-Linear Iterative Partial Least Squares*). NIPALS descompone X e Y simultáneamente en un producto de otras dos matrices de *scores* y *loadings*.

Este método es descrito por las siguientes ecuaciones:

$$\mathbf{X}=\mathbf{T}\mathbf{P}^{\mathbf{T}}+\mathbf{E}$$

$$\mathbf{Y}=\mathbf{U}\mathbf{Q}^{\mathbf{T}}+\mathbf{F}$$

donde **X** es una matriz de *N x M* predictores; **Y** es una matriz de *N x P* respuestas; $\mathbf{TP}^{\mathbf{T}}$ se aproxima a los datos de la matriz **X** y $\mathbf{UQ}^{\mathbf{T}}$ a los verdaderos valores de **Y**. La descomposición no es independiente y existe una relación lineal entre los scores **T** y **U**, que son matrices *N* x *L* (*scores*), que son a su vez, proyecciones de **X** y de **Y** respectivamente. **P** y **Q** son, respectivamente, matrices de cargas ortogonales *(loadings*); y los términos **E** y **F** de las ecuaciones anteriores son matrices de error y el superíndice T significa la transpuesta de la matriz (Wold & Sjöström, 2001).

NIPALS trata de encontrar factores (llamados variables latentes) que maximizan la cantidad de variación explicada en **X** que es relevante para la predicción de **Y**, es decir, capturar varianza y conseguir correlación. La parte importante de cualquier regresión es su uso para predecir el bloque dependiente del bloque independiente, esto se hace descomponiendo el bloque **X** y construyendo el bloque **Y** utilizando los valores calculados para la calibración.

El número de variables latentes que se utilizarán es un parámetro importante de un modelo PLS. Así, si el modelo para la relación entre **X** e **Y** es un modelo lineal, el número de componentes necesario para describir es igual a la dimensionalidad del modelo. Para la correcta selección de estos componentes, existen diversos métodos como el uso de la suma residual de

predicción de cuadrados (PRESS) o el llamado RMSECV (error cuadrático medio en la validación cruzada) (Geladi & Kowalski, 1986; Wold, Sjostrom, & Eriksson, 2001).

Esta metodología ha sido aplicada en el ámbito de control de alimentos para múltiples predicciones, como por ejemplo determinar el amargor de una cerveza o la acidez en vinagres (Christensen, Ladefoged, & Norgaard, 2005; Moros, Iñón, Garrigues, & De la Guardia, 2008), predecir parámetros sensoriales del vinagre balsámico italiano (Versari et al., 2011), o determinar y cuantificar la concentración de ciertos compuestos (como antocianos, ácidos orgánicos, etc.) en una matriz alimentaria o incluso detectar adulteraciones (Alamprese, Amigo, Casiraghi, & Engelsen, 2016; Regmi et al., 2012; Soriano, Pérez-Juan, Vicario, González, & Pérez-Coello, 2007).

1.5.4.1.2. Regresión de mínimos cuadrados parciales de múltiples vías (N-PLS)

N-PLS es un método de regresión linear de múltiples vías, siendo una extensión del método PLS, anteriormente descrito, a datos de múltiples dimensiones, es decir, a matrices de orden superior, principalmente matrices de tres dimensiones (Bro, 1996). Primero se desarrolló como un modelo similar a PARAFAC, demostrándose más tarde que N-PLS resultaba ser un algoritmo más rápido y que podía extenderse fácilmente a cualquier orden deseado para las matrices **X** e **Y**, así como que proporcionaba mejores resultados de predicción. Como en PLS, este método consiste en dos pasos: la descomposición de la matriz de calibración y el establecimiento de la relación (regresión) entre la matriz descompuesta de variables independientes y la variable o variables dependientes. Pero en este caso, la matriz de calibración **X** es un cubo, y es descompuesta en un conjunto de cubos de rango uno que describen la matriz X. Este método tiene la ventaja de poder hacer frente a los efectos de la matriz y a las propiedades intrínsecas de los analitos. Matemáticamente, para cada componente se construye un modelo de **X** e **Y**, a continuación, estos modelos se restan de **X** e **Y**, *y se* encuentra un nuevo conjunto de componentes de los residuales.

Este método ha resultado de utilidad en datos tridimensionales como la matriz excitación-emisión (EEM) de espectroscopía de fluorescencia multidimensional, para correlacionar fluoróforos presentes en la matriz alimentaria con parámetros de calidad (Guimet, Ferré, Boqué, Vidal, & Garcia, 2005) e incluso para relacionar datos sensoriales con datos cromatográficos (Marchetti, Bro, Durante, Cocchi, & Grandi, 2006).

1.5.4.2. Métodos de clasificación

1.5.4.2.1. Modelado suave independiente por analogía de clases (SIMCA)

SIMCA es un método de clasificación supervisado donde cada clase se modela usando un PCA de forma independiente, de forma que cada clase tiene un modelo específico descrito por un número óptimo de PCs. De esta manera, se puede construir un "espacio de la clase", cuyo volumen marca el límite entre las muestras que son descritas por el modelo como pertenecientes a la clase estudiada y las muestras que no pueden ser consideradas como pertenecientes a ella, para un nivel de confianza seleccionado. Tras construir los modelos PCA independientes, las muestras desconocidas son proyectadas en ellos. Para saber si una nueva muestra se ajusta a la clase, la información que se calcula es dividida en dos, una parte explicada por el modelo de la clase y otra que permanece en los residuales. Si los residuales de la muestra son significativamente mayores que los de la clase, la muestra es rechazada. Otra forma de interpretar los resultados obtenidos, es determinar el espacio definido por una clase dada (T^2) y su espacio residual (Q), con la finalidad de evaluar si los valores de cada muestra predicha están mejor descritos por el espacio T^2; de ser así, las muestras son clasificadas como miembros de la clase.

Este método fue originalmente propuesto por Svante Wold, (1980). Se denomina "suave" porque no asume la distribución de la variable, "independiente" porque cada categoría o clase se modela de forma independiente mediante PCA, y "por analogías de clase" porque se centra en similitudes entre objetos de la misma clase en lugar de en las diferencias entre clases. Uno de los parámetros cuya optimización es crucial es determinar el correcto número de PCs para cada PCA. Este método, a diferencia de PLS-DA y SVM que explicaremos en los siguientes apartados, permite que una muestra pueda ser clasificada en una clase, en varias clases o en ninguna clase. De este modo, una muestra desconocida solo se asigna a una clase para la cual tiene una alta probabilidad, y si la varianza residual de la muestra excede el límite superior para cada clase modelada en el conjunto de datos, la muestra no se asignará a ninguna de las clases.

Este método de clasificación ha sido también utilizado para resolver problemas de autenticidad y control de alimentos (Boffo et al., 2009; Cocchi et al., 2007; López-Feria, Cárdenas, García-Mesa, & Valcárcel, 2008; Xie, Ying, & Ying, 2009).

1.5.4.2.2. Análisis discriminante sobre mínimos cuadrados parciales (PLS-DA)

PLS-DA es un método supervisado de análisis, en concreto es discriminación que se basa en la regresión en mínimos cuadrados parciales PLS (anteriormente descrita), el cual, aunque se desarrolló inicialmente para construir modelos de predicción, se adaptó aún más para los problemas de clasificación.

En PLS-DA, la matriz **X** contiene las variables independientes que pueden ser el espectro o el cromatograma de cada muestra, mientras que la variable dependiente **Y** es una variable categórica, llamada "*dummy matrix*", que es definida por el analista y que codifica cada clase de las muestras de manera numérica (Ballabio & Consonni, 2013). Habitualmente, la matriz **Y** consta de números enteros (generalmente ceros y unos) y los valores predichos en este caso de PLS-DA para las muestras desconocidas son valores también numéricos entre cero y uno, convirtiéndose en una clase concreta según un umbral optimizado. Para convertir estos resultados numéricos en clases se utiliza el Teorema de Bayes, el cual, de manera resumida y general, vincula la probabilidad de un evento A dado B, con la probabilidad de B dado A.

PLS-DA reduce el número de variables utilizadas en el modelo, al combinar las variables en el conjunto de datos para calcular nuevas variables (llamadas variables latentes, LV) que tienen la máxima correlación con la variable de clase. Estas LV son combinaciones lineales de las variables originales y, en consecuencia, permiten la visualización gráfica y la comprensión de los diferentes patrones de datos y relaciones por puntuaciones-*scores*- de LV y *loadings*. En este caso, y al contrario de SIMCA, las muestras son siempre clasificadas en una de las clases, ya que su clasificación se basa en probabilidad. Por tanto, y de manera general, PLS-DA se puede considerar como un clasificador lineal de dos clases. Es decir, el método apunta a encontrar una línea recta que divide el espacio en dos regiones. Así, en la práctica, asumiendo que los valores de predicción siguen una distribución normal, el límite o umbral que las separa se marca donde el número de falsos positivos y negativos es el mismo.

Las ecuaciones fundamentales de PLS-DA son las siguientes:

$$\mathbf{X= TP+E}$$

$$\mathbf{C=Tq+f}$$

siendo **X** la matriz de datos inicial; **C** (también a veces llamado y) el conjunto de números discretos (0 y 1 o -1 1), por lo general de dos niveles, un nivel para el grupo A y el otro para el

resto de datos, o en un modelo de dos clases, el grupo B; **E** y **f** son residuales; **T** es la matriz de *scores y* **P** los *loadings* (Richard G. Brereton & Lloyd, 2014).

Esta metodología se aplica hoy en día de manera rutinaria por la mayoría de quimiométricos y ha sido ampliamente utilizada para problemas de clasificación y autentificación de alimentos (Alamprese et al., 2016; Callejón, Amigo, Pairo, Garmón, et al., 2012; Ong, 2008; Rohman & Man, 2010; Wu, Chen, Lin, & Tan, 2016).

1.5.4.2.3. Análisis discriminante N-PLS (NPLS-DA)

NPLS-DA o también llamado N-*way* PLS-DA, es una extensión de PLS, utilizada en el caso de datos tridimensionales. Esta metodología consiste en aplicar el algoritmo N-PLS a la clasificación, prediciendo la pertenencia de una muestra a un grupo cualitativo previamente definido (Vigneau, Qannari, Jaillais, Mazerolles, y Bertrand, 2006). En esencia, N-PLS para análisis discriminante es el mismo que para propósitos de calibración explicado anteriormente, solo que el vector Y en este caso es la *dummy matrix* o *dummy vector*, conteniendo cada clase como 1 o 0, como ocurre en el método de PLS-DA (Folch-Fortuny, Prats-Montalbán, Cubero, Blasco, & Ferrer, 2016). Igualmente, se utiliza para problemas de clasificación en matrices tridimensionales como las EEM o imagen hiperespectral (Azcarate, Teglia, Karp, Camiña, & Goicoechea, 2017; Oliveri et al., 2014).

1.5.4.2.4. Máquina de vectores de soporte (SVM)

La máquina de vectores de soporte, máquina de soporte vectorial o máquinas de vector soporte (*Support Vector Machines*, SVM) es una técnica de clasificación desarrollada por Vapnik 1979 (Vapnik, 1979) basada en un conjunto de algoritmos de aprendizaje supervisado.

SVM es un método de clasificación bastante reciente que no necesita un gran número de muestras para ser entrenado y no se ve afectado por la presencia de valores atípicos. Además, SVM está ganando interés con respecto a otras técnicas de clasificación debido a su capacidad para realizar clasificaciones lineales y no lineales.

El problema de clasificación se puede restringir a un problema de dos clases. Así, dado un conjunto de muestras de entrenamiento, podemos etiquetar las clases y entrenar al modelo SVM para predecir la clase de una nueva muestra. Una SVM es un modelo que representa a las muestras como puntos en el espacio, separando las clases a 2 espacios lo más amplios posibles mediante un hiperplano de separación óptimo (OSH) definido como el vector entre los 2 puntos más cercanos, de las 2 clases, a los que se le llaman “vectores soporte”. Cuando las nuevas

muestras se ponen en dicho modelo, en función de los espacios a los que pertenezcan, pueden ser clasificadas a una o la otra clase. Por tanto, en resumen, SVM busca encontrar un hiperplano que separe de forma óptima las diferentes clases involucradas. Esto se hace maximizando la distancia entre el hiperplano y las muestras más cercanas del conjunto de entrenamiento (los vectores de soporte). En ese concepto de "separación óptima" es donde reside la característica fundamental de SVM: este tipo de algoritmos buscan el hiperplano que tenga la máxima distancia (margen) con los puntos que estén más cerca de él mismo. Los llamados vectores de soporte son los puntos que tocan el límite del margen del hiperplano. Cuando los datos no se pueden separar linealmente se hace un cambio de espacio mediante una función que transforme los datos de manera que se puedan separar linealmente. Tal función se llama Kernel y en este caso se incluye un nuevo parámetro llamado "coste de error" *C*. SVM se han aplicado con éxito a varios problemas de clasificación en el ámbito agro-alimentario (Haddi et al., 2013; Osuna, Freund, & Girosi, 1997; Zhao et al., 2010).

1.5.4.2.5. Modelo de clasificación jerárquica (HCM)

La clasificación es un proceso que consiste en asociar una muestra particular a una o más clases entre un conjunto de clases predefinidas de acuerdo con las características propias de la muestra. En general, la clasificación se puede dividir en dos tipos: clasificación plana o convencional y clasificación jerárquica (Borges, Silla, & Nievola, 2013). La clasificación jerárquica o *Hierarchical multi-label classification* (HMC) es una variante de clasificación en la que una muestra puede pertenecer a varias clases al mismo tiempo las cuales clases están organizadas en una jerarquía, como un árbol de categorías. La organización en jerarquía significa que un objeto que pertenece a alguna clase pertenece automáticamente a todas sus superclases (Vens, Struyf, Schietgat, Džeroski, & Blockeel, 2008). En la práctica, muchos problemas de clasificación importantes necesitan sistemas de clasificación jerárquica, como la taxonomía, en la que un objeto pertenece sucesivamente a una especie, un género, una familia y un orden (Silla & Freitas, 2011). Sin embargo, la mayoría de los enfoques encontrados en la literatura ignoran la estructura jerárquica y tratan cada categoría o clase por separado (es decir, cada clase es independiente de las demás) por lo que "aplanan" la estructura de una clase, es decir, utilizan una clasificación plana (Dumais & Chen, 2000). Mediante el uso de una estructura jerárquica, un problema complejo de clasificación puede ser descompuesto en un conjunto de problemas más pequeños correspondientes a divisiones jerárquicas en estructura de árbol. Así, cada uno de estos sub-problemas se pueden resolver de manera mucho más eficiente y fácil. Esto es debido a que cada sub-problema es más pequeño que el problema original y por tanto necesita un

conjunto de funciones menor para resolver cada uno. A pesar de las ventajas que presenta una clasificación jerárquica, no hay muchos estudios que hayan implementado esta clasificación en términos de clasificación de alimentos (Beckonert et al., 2003; Dupuy, Galtier, Ollivier, Vanloot, & Artaud, 2010; Hossain, Patras, Barry-Ryan, Martin-Diana, & Brunton, 2011).

Como se muestra en la Figura 21, hay diferentes tipos de enfoques de clasificación jerárquica: uno es el llamado "enfoque de clasificación plana" (*Flat classification approach*), que es el más simple y consiste en ignorar completamente la jerarquía de clases, generalmente buscando solo las clases en los nodos (o clases) finales de clasificación, es decir del nivel más bajo de clases descrito. El otro enfoque, más utilizado en la literatura, es el "clasificador local por nodo" *(Local classifier per node*-LCN), que consiste en entrenar un clasificador binario para cada nodo de la jerarquía de clases excepto para el primer nodo o clase (llamado root node), y el "clasificador local por nodo primario o padre" (*Local classifier per parent node*-LCPN) que entrena un clasificador multi-clase para cada nodo-padre en la jerarquía de clases para distinguir entre sus nodos secundarios-hijos, incluyendo una clasificación en el *root node* (Silla & Freitas, 2011).

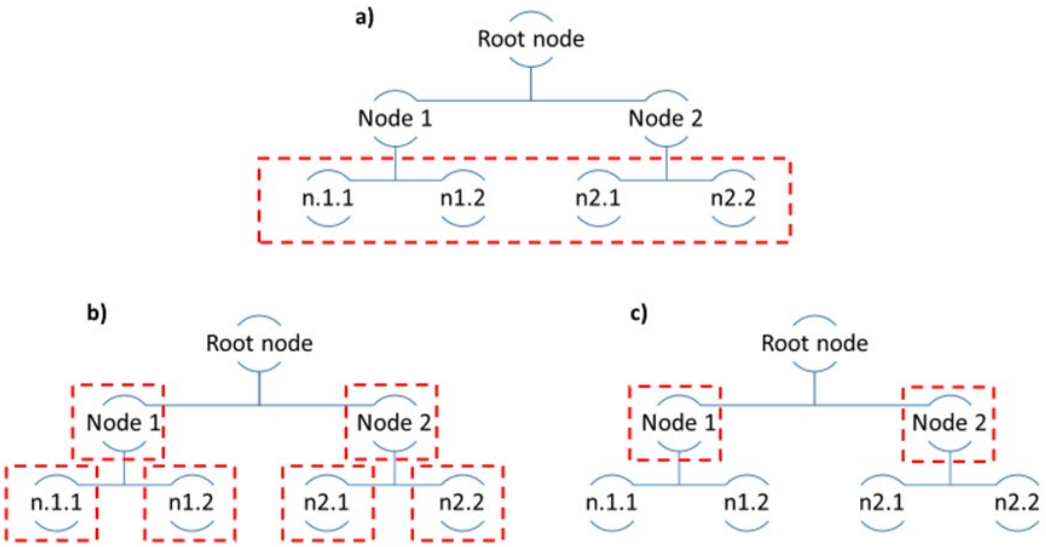


Figura 21. Esquema de tres tipos de clasificación jerárquica: a) *Flat classification approach,* b) *Local classifier per node*, c) *Local classifier per parent node*. Los círculos representan las clases o grupos y los cuadrados rojos representan: a) los últimos nodos predichos; b) clasificadores binarios; y c) los clasificadores multi-clase.

1.5.5. TÉCNICAS DE FUSIÓN DE DATOS

La calidad de los alimentos se basa en una combinación compleja de diferentes características, por lo que las mediciones analíticas para un único compuesto o por una única técnica rara vez pueden correlacionarse completamente con el cumplimiento de la calidad. Así, para determinar la calidad de un alimento sería necesario realizar un análisis de datos multivariados, que nos permitiría tanto obtener la información de calidad más completa, como monitorear los parámetros clave de producción. En esta progresión de mejorar la evaluación de la calidad y la autentificación de los alimentos, un paso más avanzado es combinar los resultados de múltiples fuentes instrumentales, como por ejemplo una combinación de las técnicas de sensores, cromatografía y espectroscopía rápidas y fiables. Esto ha provocado una evolución enorme del análisis de datos en los últimos años y dentro de este contexto, surge la metodología de fusión de datos que, hoy en día, se encuentra en creciente desarrollo. Esta metodología consiste básicamente en la combinación de datos procedentes de múltiples fuentes. Así, la fusión de datos de técnicas complementarias puede proporcionar un conocimiento más preciso sobre una muestra y producir clasificaciones con menos tasa de error y predicciones con menos incertidumbre que una sola técnica.

El concepto de fusión de datos en la autentificación de alimentos no es nuevo, ya que los seres humanos combinan múltiples sentidos para lograr describir la calidad e idoneidad de los alimentos. Y tampoco es nuevo para los quimiometristas, que durante mucho tiempo han combinado parámetros químicos individuales determinados por análisis clásicos o instrumentales en una matriz única con el objetivo de mejorar los resultados de la autentificación alimentaria. Pero el desafío de hoy en día consiste en combinar de forma significativa no solo variables individuales como se hizo en el pasado, sino combinar bloques de variables.

La fusión de datos requiere desarrollar nuevas ideas para pre-procesar y unir bloques de datos, seleccionar variables y validar modelos. Por último, pero no menos importante, los datos a fusionar deben proporcionar información complementaria para ser útiles. Esto significa que el conocimiento químico sobre las muestras y el problema en cuestión es fundamental para seleccionar las técnicas analíticas adecuadas. Estas técnicas además ya han demostrado ser, en trabajos recientes, una herramienta simple y poderosa, que contribuye al análisis de datos complejos registrados por diferentes técnicas, permitiendo una mayor extracción de información y la construcción de modelos más eficaces en algunas matrices alimentarias (Alamprese, Casale, Sinelli, Lanteri, & Casiraghi, 2013; Biancolillo, Bucci, Magrì, Magrì, & Marini,

2014; Borràs et al., 2015; Silvestri et al., 2014; Silvestri et al., 2013). Las técnicas de fusión de datos se clasifican en tres niveles: nivel bajo, medio y alto. A continuación, se describen un poco más cada uno de los niveles: nivel bajo, medio y alto.

El nivel bajo de fusión de datos es el más simple. Consiste en la simple concatenación de datos de distinta naturaleza, teniendo en cuenta el pre-procesado y/o normalización de los datos por separado o del conjunto. En la Figura 22 se muestra un ejemplo práctico de este nivel de fusión de datos de dos matrices de datos espectroscópicos.

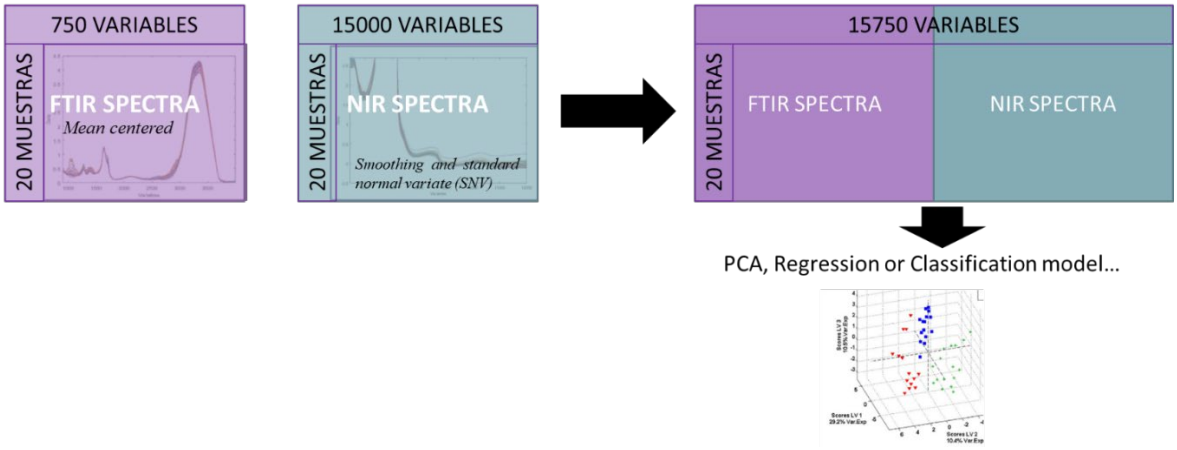


Figura 22. Ejemplo esquemático de una fusión de datos analíticos de nivel bajo.

En el nivel medio se fusionan los datos que han sido previamente extraídos de los datos originales individualmente. Es decir, previo a la fusión, se realiza alguna técnica para la reducción de datos como técnicas multivariantes (ej. PCA, MCR, PARAFAC…) como estrategias de selección de variables. Estos datos reducidos se unen o concatenan y se aplican técnicas de regresión o clasificación sobre ellos (Figura 23). Dentro de este nivel se encuentra una metodología novedosa conocida como P-Comdim, que proporciona las fuentes comunes de información compartidas por cada bloque de datos, es decir, los componentes comunes, al mismo tiempo que asigna a cada bloque individual un peso específico (o saliente) asociado a cada dimensión del espacio común (Cariou, Qannari, Rutledge, & Vigneau, 2016; Ghaziri, Cariou, Rutledge, & Qannari, 2016). Este método se ha aplicado recientemente al análisis de varios productos alimentarios (Erich et al., 2015; Hohmann et al., 2015; Mazerolles et al., 2002).

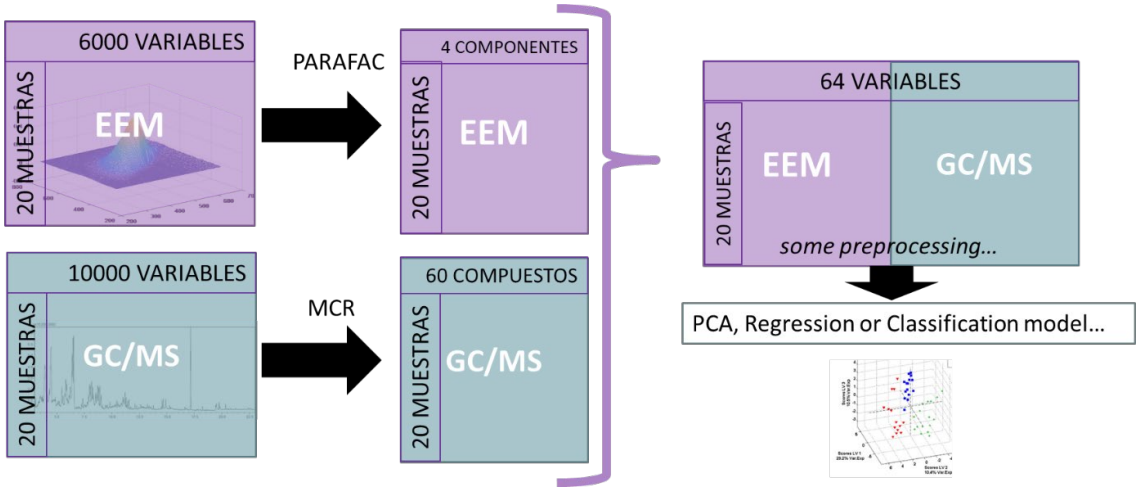


Figura 23. Ejemplo esquemático de fusión de datos de nivel medio.

En el caso del nivel de fusión de datos alto, se hacen modelos previos para cada set de datos individualmente (modelos como PLS-DA, SVM, SIMCA…) y después, las respuestas de estos modelos se unen para producir la respuesta final fusionada a la cual se le puede aplicar algún tratamiento de datos multivariante para obtener resultados de regresión o clasificación, como muestra la Figura 24.

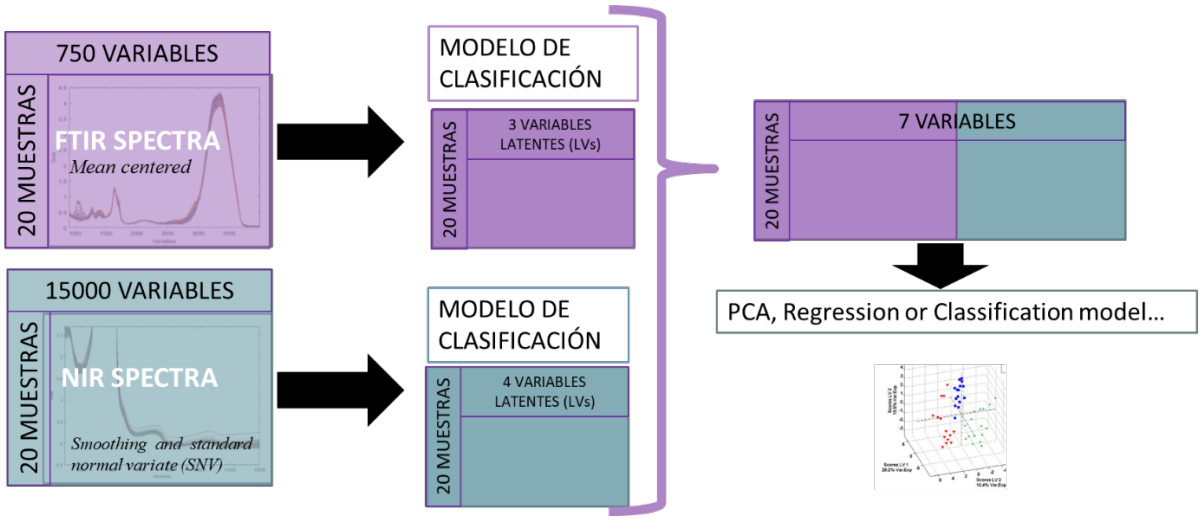


Figura 24. Ejemplo esquemático de fusión de datos de nivel alto.

Las desventajas o inconvenientes que muestran estás técnicas de fusión de datos es que es un campo poco estudiado aún, en el que no existen demasiadas reglas escritas de cómo y qué se puede hacer. Así, es difícil saber qué nivel de fusión de datos necesita un problema analítico sin realizar pruebas, empezando normalmente por nivel bajo y subiendo a los demás niveles si los resultados no son lo suficientemente adecuados. Además, la fusión de datos no solo consiste en la unión directa de dos tablas, sino que se necesitan pre-procesados especiales tanto de los

datos por separado como de los datos una vez unidos. Por otro lado, hay que tener en cuenta que no siempre la fusión de datos es necesaria, ya que si con unos datos individuales se pueden obtener buenos resultados o los resultados que buscamos, no tendría sentido el uso de las técnicas de fusión de datos.

1.5.6. MÉTODO DE REMUESTREO-*BOOTSTRAPPING*

En el ámbito de la estadística, el método de remuestreo o *resampling* se le denomina a los métodos que permiten estimar la precisión de muestras estadísticas mediante el uso de subconjuntos de datos (*jackknifing*) o tomando datos de forma aleatoria de un conjunto de datos (*bootstrapping*), así como a los que permiten realizar test de significancia o validad modelos.

En 1979, Bradley Efron (Efron, 1979) desarrolla y publica el análisis de remuestreo *Bootstrap*. En la actualidad, los procedimientos de remuestreo o *bootstrapping* son ampliamente conocidos y utilizados en diversos campos de la investigación para de manera general, estimar los parámetros y/o la incertidumbre de un modelo siendo la estimación de la incertidumbre un parámetro importante para evaluar en datos analíticos. La idea principal es usar datos para generar más datos. Así, el término de remuestreo es aplicado a aquellas técnicas de simulación empleadas en la teoría de probabilidades e inferencia estadística que, a partir de datos observados, generan nuevas muestras simuladas con el objetivo de examinar los resultados obtenidos para estas muestras. En el caso concreto de *bootstrapping,* las nuevas muestras simuladas se consiguen por la técnica del reemplazo, de tal modo que de una muestra se seleccionaran algunos elementos y otros no. *Bootstrapping* es una técnica de remuestreo que hace posible resolver tareas difíciles cuando el tamaño de las muestras es muy pequeño o cuando hay muchos niveles de clasificación en la estructura, como la obtención de intervalos de confianza, pruebas de significación estadística o cualquier otra estadística. La idea es generar múltiples conjuntos de datos que, después del análisis, muestren la variabilidad estadística de interés. Para eso, *bootstrapping* extrae muestras sucesivas de una muestra real volviendo a muestrear mediante reemplazo o reposición, para que algunos elementos no se seleccionen y otros se puedan seleccionar más de una vez en cada muestreo para hacer nuevas muestras simuladas. Así, las nuevas muestras serán algo diferentes de la muestra original, con lo cual un estadístico calculado a partir de una de las nuevas muestras tendrá un valor diferente del de otra muestra nueva.

Mediante el método *Bootstrap*, se consiguen estimar los errores estándar de los datos originales mediante un nuevo muestreo, lo que permite calcular los intervalos de confianza para cada muestra, así como la confiabilidad en los modelos de clasificación al conocer la incertidumbre de cada grupo definido en el modelo jerárquico (Snee, 1977). Además, *Bootstrap* proporciona límites de confianza consistentes (CL), que son la manera más común de estimar la incertidumbre de un modelo. Con la distribución *Bootstrap*, el valor central (el punto estimado) y los intervalos de confianza (límites de confianza) se pueden calcular de manera similar al cálculo del intervalo de confianza de una media. Por tanto, en resumen, este método permite valorar el error muestral, establecer un intervalo de confianza y validar modelos.

2. JUSTIFICACIÓN Y OBJETIVOS

JUSTIFICATION AND OBJECTIVES

JUSTUFICACIÓN

En la actualidad, cada vez son más frecuentes las noticias relacionadas con fraudes alimentarios y falsificaciones, sobretodo en productos alimenticios de alta calidad. Esto ha provocado que la autenticidad de estos alimentos sea una preocupación que está cobrando una mayor importancia en la vida diaria de productores, consumidores e investigadores.

Este interés social ha dado lugar al nacimiento de nuevas normativas y regulaciones de los países europeos y de Estados Unidos, generándose nuevos retos analíticos para los laboratorios de control de la calidad de los alimentos. La autenticidad y la lucha contra el fraude es uno de los objetivos promotores de la Unión Europea (UE), así como una de las líneas de actuación del Programa Marco de Investigación e Innovación de la UE (Horizonte 2020) calificado como “desafío de la sociedad”.

La autentificación de alimentos comprende el control de un amplio rango de parámetros fisicoquímicos y sensoriales. Los métodos comúnmente usados para caracterizar, evaluar la calidad o descubrir adulteraciones y fraudes en alimentos, son principalmente la cromatografía de gases o de líquidos acoplados a espectrometría de masas (GC-MS, HPLS-MS) o electroforesis capilar (CE). Éstos tienen la desventaja de que consumen bastante tiempo, son caros, laboriosos y requieren entrenamiento del personal. Así mismo, los compuestos que deben ser cuantificados para asegurar la autenticidad alimentaria están continuamente aumentando debido a la mayor sofisticación de los métodos de adulteración. En consecuencia, hay una creciente necesidad de buscar métodos analíticos rápidos, sencillos, baratos, robustos, efectivos y que no requieran apenas manipulación de la muestra, capaces de autentificar alimentos, clasificarlos y detectar adulteraciones o fraudes. Con esta finalidad, las técnicas espectroscópicas combinadas con técnicas quimiométricas, han demostrado ser útiles para la caracterización y autentificación de alimentos, así como para la detección de posibles adulteraciones o fraudes.

Hoy en día, uno de los productos alimenticios que se están viendo afectados por numerosos fraudes y falsificaciones son aquellos que se encuentran protegidos bajo una Denominación de Origen Protegida (DOP). Dicha protección sirve para garantizar al consumidor un nivel de calidad elevado y constante y unas características singulares de dichos productos elaborados en una zona geográfica específica. Además, otorga a los productores una protección legal contra falsificaciones. Entre los productos amparados bajo una DOP, encontramos el vinagre de vino. En España son tres las DOP de vinagre de vino reconocidas: “Vinagre de Jerez”,

"Vinagre del Condado de Huelva", y "Vinagre de Montilla-Moriles", siendo las tres producidas en Andalucía. La alta calidad de estos vinagres de vino con DOP se debe fundamentalmente a su aroma, ya que es lo que el consumidor percibe como calidad. Así, estos vinagres de vino con DOP cuentan con una complejidad aromática y sensorial originados e influenciados por el material de partida, el método de producción, los compuestos formados durante la fermentación, y en algunos casos, durante el envejecimiento. Por tanto, uno de los parámetros principales necesarios para determinar la calidad de estos vinagres y diferenciarlos de otros y entre sí, es el estudio de su perfil volátil, aromático y sensorial, el cual no se había realizado hasta la fecha con los vinagres de vino de las tres DOP españolas.

En este contexto, el proyecto de tesis titulado "Caracterización espectroscópica y aromática de vinagres españoles con denominación de origen protegida" pretende, por un lado, ofrecer una nueva metodología analítica que permita de forma rápida y eficaz la caracterización y detección de fraudes en alimentos con un alto valor añadido en la dieta Mediterránea, como son los vinagres con DOP, para garantizar que el consumidor reciba un producto con total garantía en cuanto a seguridad, origen y métodos de producción, utilizando un método de control económico, rápido y sencillo como alternativa a los métodos tradicionales. Para la consecución de este objetivo se propone emplear técnicas espectroscópicas en combinación con técnicas quimiométricas, debido a que esta combinación ya ha demostrado ser altamente competitivas en el campo de la caracterización y clasificación de alimentos por ser técnicas no destructivas, sensibles, rápidas y de relativo bajo coste. Por otro lado, el proyecto de tesis también pretende realizar una caracterización aromática de estos vinagres de vino con DOP mediante la determinación de sus perfiles volátiles, aromáticos y sensoriales, así como la determinación de los compuestos aromáticos que puedan servir como marcadores de calidad y autenticidad de cada DOP.

OBJETIVOS

Uno de los objetivos principales del proyecto de Tesis es la **caracterización espectroscópica** de los vinagres de **vino españoles con DOP** mediante métodos analíticos más rápidos y económicos que los métodos tradicionales, que permitan la autentificación y discriminación de los vinagres de las 3 DOP españolas (Vinagre de Jerez, Vinagre de Condado de Huelva y Vinagre de Montilla-Moriles), y de sus categorías, garantizando con ello su **autenticidad**, su **denominación de origen** y la **categoría** a la que pertenecen. Además, por otro lado, el segundo objetivo principal es la **caracterización aromática** de los vinagres de vino de las tres DOP y sus categorías mediante el estudio de su **perfil aromático** por **cromatografía de gases-espectrometría de masas, análisis olfatométricos y análisis sensorial**, con el objeto de intentar relacionar los datos sensoriales con los obtenidos instrumentalmente y obtener con todo ello una caracterización aromática completa de las muestras que permita su autenticación y discriminación. Todo esto se culminaría con el desarrollo de una **herramienta informática** basada en los resultados obtenidos en combinación con **métodos de análisis multivariante**, que sea capaz de conseguir los objetivos citados.

Para ello se definen los siguientes sub-objetivos:

1. Caracterización de vinagres de vino españoles con DOP mediante **técnicas espectroscópicas** rápidas, económicas y robustas (EFM, FTIR, NIR, RMN, UV-VIS) en combinación con técnicas quimiométricas.

2. Construcción de **modelos multivariantes de clasificación** a partir de los resultados espectroscópicos, **individualmente o de forma fusionada**, que permitan la diferenciación y autenticación entre DOP, así como entre las distintas categorías comercializadas según tiempo de envejecimiento y dulzor, e incluso la detección de adulteraciones o de prácticas fraudulentas.

3. Caracterización aromática de los vinagres de vino con DOP mediante **análisis GC-MS, análisis sensorial y análisis olfatométrico (GC-MS-O),** con el fin de establecer las diferencias entre el perfil volátil y aromático de las diferentes DOPs y categorías, para darle un mayor valor añadido a estos vinagres y DOPs, así como determinar aquellos compuestos volátiles y odorantes de impacto responsables de la diferenciación de estos vinagres, los cuales podrían considerarse marcadores de la calidad y autenticidad de cada DOP y categoría.

4. Construcción de una **herramienta informática** basada en **métodos de análisis multivariante** a partir del mejor modelo de clasificación que, de forma rápida y sencilla, permita **identificar los vinagres de vino de distintos orígenes, distintos métodos de producción, distintos envejecimientos, así como vinagres de vino con o sin D.O.P,** garantizando la autenticidad de cada vinagre y detectando muestras fraudulentas, siendo susceptible de patente.

La realización de la presente propuesta requiere una experiencia multidisciplinar y complementaria. Está vinculada a un proyecto de Excelencia de la Junta de Andalucía (P12-AGR-1601), el cual ha sido dirigido desde la Universidad de Sevilla y en el que participan tres centros de investigación (Universidad de Sevilla, Instituto de la Grasa y Universidad de Copenhague). El conocimiento generado permitirá dar un valor añadido a la producción y comercialización de vinagres andaluces con DOP. Además, todos estos objetivos específicos se enmarcan en un objetivo general de la industria vinagrera centrada en la obtención de procedimientos de trazabilidad integral, aumentando de esa forma la confianza del consumidor y reforzando la competitividad de las empresas en un mercado agroalimentario cada vez más competitivo.

DIAGRAMA DE FLUJO

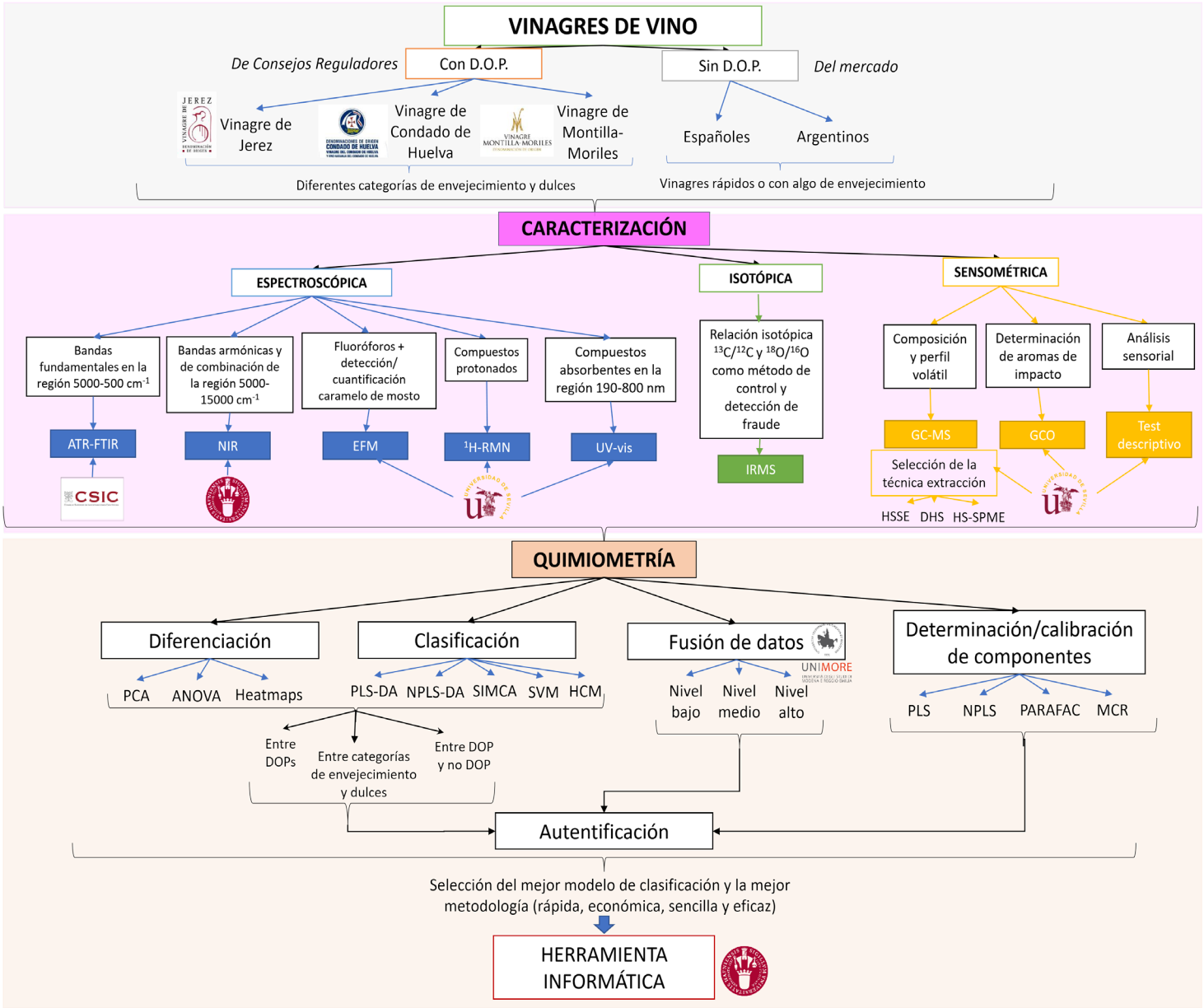


Figura 25. Diagrama de flujo de los objetivos a realizar en la presente Tesis Doctoral.

3. MATERIALES Y MÉTODOS

MATERIALS AND METHODS

3.1. MUESTRAS

Para el desarrollo de la presente tesis doctoral se recolectaron muestras de vinagres de vino españoles con DOP (Vinagre de Jerez, Vinagre de Montilla-Moriles y Vinagre de Condado de Huelva), pertenecientes a las distintas categorías registradas de envejecimiento y dulces o semi-dulces. Estas muestras fueron proporcionadas por los Consejos Reguladores de cada una de las DOP en distintos años, consiguiendo las mismas muestras, pero de distintos lotes. Además, se compraron muestras de estas DOP en supermercados para ampliar el número de muestras y realizar una comparación entre las del Consejo Regulador y las que encontramos en el mercado.

Además, por otro lado, se compraron muestras de vinagres de vino sin la indicación DOP, obteniéndolas de distintos supermercados e incluso bodegas de distintas regiones del país. Finalmente, gracias a una colaboración con el CONICET de Argentina, se añadieron en el último año de esta presente tesis, muestras de vinagres de vino de origen argentino, tanto de supermercados como procedentes de bodegas de allí. A pesar de que ninguna de estas muestras tenía la indicación de DOP, algunas se produjeron de forma tradicional en bodegas envejeciéndolas por un cierto periodo de tiempo.

Un resumen de las muestras obtenidas y analizadas por los distintos métodos puede verse en la siguiente **Tabla 6**. Como puede observarse, no todas fueron analizadas por todos los métodos, debido a que las botellas se gastaban, las muestras se estropeaban o fueron proporcionadas con posterioridad al análisis en cuestión.

Tabla 6. Resumen del total de muestras de vinagre de vino analizadas en el presente trabajo de tesis por distintas técnicas analíticas.

TÉCNICAS / CATEGORÍA	MUESTRA	FTIR	NIR	FLUORESCENCIA	UV-VIS	GC-MS	RMN	ISOTOPOS	GCO
DOP VINAGRE DE CONDADO DE HUELVA									
SIN CRIANZA	CSC_DIA1,2,3	X	X	X	X	X	X	X	
	CSC_AND1	X	X	X	X	X	X	X	
	CSC_JML1	X	X	X	X	X	X	X	
	CSC_RAP1,2	X	X	X	X	X	X	X	
	CSC_CML1,2,3	X	X	X	X	X	X	X	
SOLERA	CSO_CML1	X	X	X	X	X	X	X	
	CSO_JML1	X	X	X	X	X	X	X	
	CSO_TOC1,2	X	X	X	X	X	X	X	
	CSO_DIA1	X	X	X	X	X	X	X	
	CSO_RAP1,2	X	X	X	X	X	X	X	
RESERVA	CRE_AND1	X	X	X	X	X	X	X	
	CRE_BOT1,2	X	X	X	X	X	X	X	
	CRE_JML1,2	X	X	X	X	X	X	X	
	CRE_RUBC1,2,3	X	X	X	X	X	X	X	
	CRE_CML1	X	X	X	X	X	X	X	X
	CRE_DIA1,2	X	X	X	X	X	X		
	CRE_RAP1	X	X	X		X	X		
AÑADA	CAN_CML1	X	X	X			X		
	CAN_CML2	X	X	X			X		
	CAN_RUB1,2	X	X	X			X		
DOP VINAGRE DE JEREZ									
CRIANZA	JCR_VRL1	X	X	X		X	X	X	
	JCR_GCL1	X	X	X		X	X	X	
	JCR_CAR1	X	X	X	X	X	X	X	
	JCR_BAD1	X	X	X	X	X	X	X	
	JCR_BOR1	X	X	X	X	X	X	X	
	JCR_TPL1	X	X	X	X	X	X	X	
	JCR_MRL1	X	X	X		X	X	X	
	JCR_PAR1	X	X	X			X		
	JCR_PET1	X	X	X			X		
	JCR_SRL1	X	X	X	X		X	X	
	JCR_JPL1	X	X	X					
	JCR_CAP1	X					X	X	
	JCR_RSL1				X		X	X	
	JCR_SOL77						X	X	
	JCR_AOL1	X	X	X			X	X	
	JCR_EML1	X	X	X			X		
	JCR_PAES								
	JCR_BORD1		X						
	JCR_DIA1		X	X	X		X	X	
RESERVA	JRE_VRL1	X	X	X	X	X	X	X	
	JRE_BAR1	X	X	X	X	X	X	X	X
	JRE_BDL1	X	X	X	X	X	X	X	
	JRE_ONE1	X	X	X	X	X	X	X	
	JRE_SAN1	X	X	X	X	X	X	X	
	JRE_YBA1	X	X	X	X	X	X	X	
	JRE_ARV1	X	X	X	X	X	X	X	
	JRE_RSL1	X	X	X		X	X		
	JRE_GAR1	X	X	X			X		
	JRE_JPL1	X	X	X			X		
	JRE_FOL1	X	X	X			X		
	JRE_VAL1	X	X	X			X		
	JRE_PAES		X						

	JRE_CEN		X				X		
	JRE_EPA1		X						
GRAN RESERVA	JGR_GBL1/2	X	X	X			X		
	JGR_EML	X	X	X					
Pedro Ximénez	JPX_SRL2	X	X	X		X	X	X	
	JPX_LUS1	X	X	X		X	X	X	X
	JPX_EML1	X	X	X		X	X	X	
	JPX_DBL1	X	X	X		X	X	X	
	JPX_GCL	X	X			X	X	X	
	JPX(RE)_LUS					X	X	X	
DOP VINAGRE DE MONTILLA-MORILES									
CRIANZA	MCR_CCL1	X	X	X	X	X	X	X	
	MCR_AUR1	X	X	X	X	X	X	X	
	MCR_JNL1	X	X	X	X	X	X	X	
	MCR_UNI1	X	X	X	X	X	X	X	
	MCR_ROB1	X	X	X	X	X	X	X	
	MCR_ROB4	X	X	X			X		
	MCR_CVS7	X	X	X			X	X	
RESERVA	MRE_AUR1	X	X	X	X	X			
	MRE_UNI1	X	X	X	X	X			
	MTR_CVS6	X	X	X			X		
	MRE_CVS8	X	X	X	X	X	X	X	X
	MRE_CVS9	X	X	X	X	X	X	X	
	MGR_CCL1	x	X	X	X	X	X	X	
	MSO_LOL	x	X	X	X	X	X	X	
Pedro Ximénez	MPX_ALV1	X	X	X	X	X	X	X	X
	MPX_CCL1	X	X	X		X	X	X	
	MPX_UNI1	X	X	X		X	X	X	
	MPX_ROB2	X	X	X		X	X	X	
	MPX_ROB3	X	X				X	X	
VINAGRES SIN DOP O RÁPIDOS									
NO-DOP ESPAÑA	VPX_DIA1		X						
	VR_CARD1		X		X				
	VPX_EPA1		X						
	VPX_TA1		X						
	VJ_COH1		X						
	VR_SUP		X		X				
	GAL-OLE 2015		X					X	
	GAL-ECI 2015		X					X	
	CAT-GAR 2015		X					X	
	CAT-FOR 2015		X		X			X	
	CAT-BOR 2015		X		X			X	
	RIO-RIO 2015		X		X			X	
	RIO-ALI 2015		X		X			X	
	RIO-FEM 2015		X		X			X	
NO-DOP ARGENTINA	ALC_18				X				
	LA_17				X				
	MEN_24				X				
	CAR_23				X				
	CAS_14				X				
	COM_16				X				
	CON_22				X				
	DOS_20				X				
	FAV_25				X				
	GRE_15				X				
	HAL_21				X				

3.2. MATERIALES, INSTRUMENTACIÓN Y PROCEDIMIENTOS ANALÍTICOS

3.2.1. ANÁLISIS ESPECTROSCÓPICO

3.2.1.1. Espectroscopía de infrarrojo medio con Transformada de Fourier y cristal de Reflactancia total atenuada (ATR-FTIR)

- Materiales y reactivos:
  - Espectrómetro Bruker Vertex 70 FTIR equipado con detector DGTS (Bruker Optics, Ettlingen, Alemania)
  - Accesorio ATR: La estación de muestreo estaba equipada con un accesorio de reflectancia total atenuada y multi-reflexión desmontable (ATR, seis rebotes, Specac, Orpington, U.K.). Este accesorio consiste en un cristal ZnSe de 45°C de ángulo.
  - Ácido acético de calidad analítica - Merck (Madrid, España)
  - Agua Milli-Q (Millipore, USA)

- Instrumentación y procedimiento analítico:

El análisis se llevó a cabo en el espectrómetro que se muestra en la Figura 26. Cada espectro se registró a la misma temperatura (22°C) en la región de 4000-600 cm^{-1} con un promedio de 50 exploraciones a una resolución de 4 cm^{-1}. Antes de analizar cada muestra, se tomó un espectro de fondo con un cristal ATR vacío. 250 µl de cada muestra se extendieron uniformemente a través del cristal ATR utilizando una micropipeta. Los espectros se examinaron utilizando OPUS versión 7.0 (Bruker Optics, Ettlingen, Alemania) y se manipularon con el software OMNIC. Cada muestra fue analizada por triplicado. Estos análisis se llevaron a cabo en el Instituto de la Grasa, CSIC.

Figura 26. Imágenes del equipo utilizado para el análisis de las muestras por ATR-FTIR.

3.2.1.2. Espectroscopía de Infrarrojo cercano (NIR)

- Materiales y reactivos:
  - Espectrómetro IR ABB de Bomen (Q-interline, X, Dinamarca), equipado con una longitud de trayectoria de 1 mm para cubetas.
  - Vial transparente de 1 mL, 40x80 mm (Skandinaviska Genetec AB, Lund, Suencia)
  - Ordenador con software de espectroscopía GRAMS/AI™ (software Thermo Fisher Scientific)

- Instrumentación y procedimiento analítico:

Los datos espectrales se recopilaron tras el análisis de los vinagres de vino por el espectrómetro mostrado en la Figura 27, en el rango de 12000-4000 cm^{-1}, con una resolución de 8 cm^{-1} y 64 exploraciones. Las muestras de vinagre de vino se analizaron directamente sin tratamiento previo de la muestra pipeteándolas en un vial transparente de 1 ml antes de su análisis. El espectro de cada muestra se obtuvo por triplicado en una secuencia aleatoria y a temperatura ambiente (21-23 °C). Estos análisis se llevaron a cabo en el Departamento de Ciencia de los Alimentos de la Universidad de Copenhague.

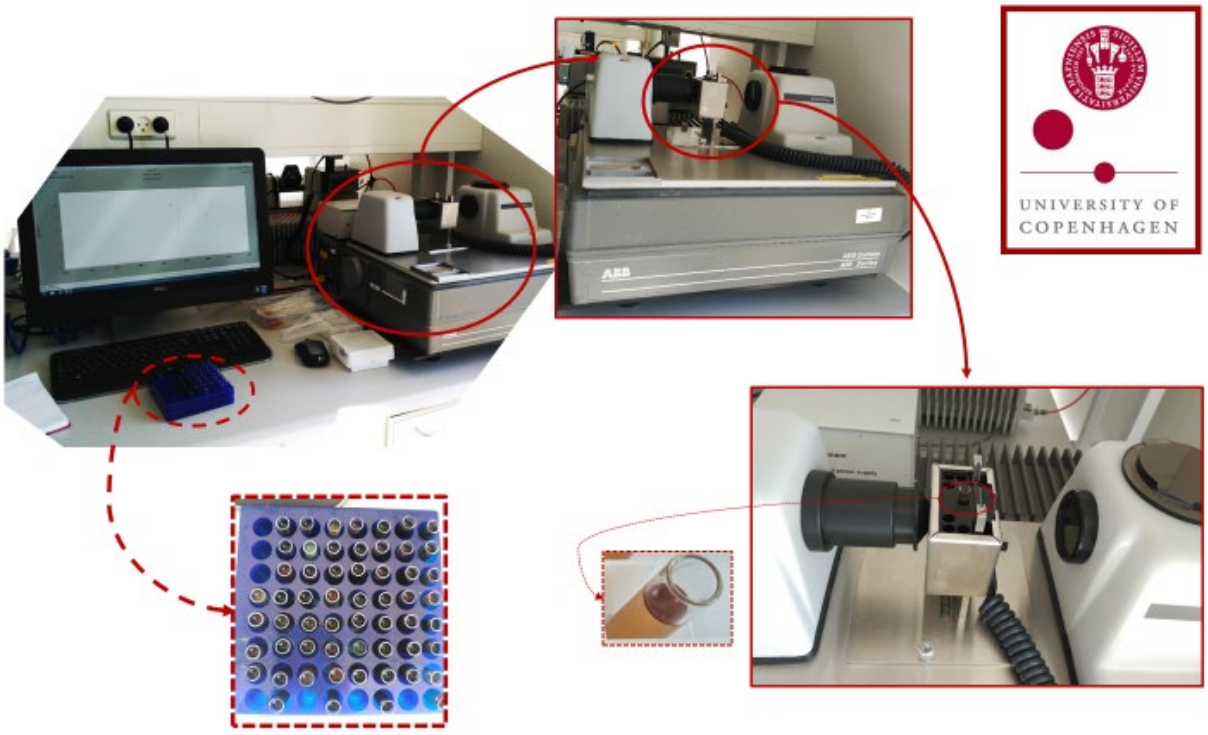


Figura 27. Imágenes del equipo NIR y cubetas utilizadas para el análisis de las muestras de vinagre de vino.

3.2.1.3. Espectroscopía de fluorescencia multidimensional (EFM)

3.2.1.3.1 Determinación de huella digital o perfil espectroscópico

- Materiales y reactivos:
  - Espectrofotómetro de fluorescencia Varian Cary-Eclipse (Varian Iberica, Madrid, España) equipado con dos monocromadores Czerny-Turner, una lámpara de descarga pulsada de xenón a 80 Hz con altura media de pico de 2 µ (poder de pico equivalente a 75 kW) y un detector de tubo fotomultiplicador R298 de alto rendimiento.
  - Cubetas de cuarzo de 3,5 ml estándar (Hellma Analytics, Müllheim, Alemania) de 1 cm de longitud de trayectoria.
  - Recipiente termostatizado Peltier (25,00 ± 0,05 °C).
  - Ordenador con software Cary-Eclipse

- Instrumentación y procedimiento analítico:

Las muestras de vinagre de vino se analizaron directamente por el espectrofotómetro de fluorescencia sin tratamiento previo de la muestra, pipeteándolas directamente en cubetas de cuarzo para su análisis. El equipo y cubetas utilizado se muestra en la siguiente figura (Figura 28). Estos análisis se llevaron a cabo en el Departamento de Química Analítica de la Facultad de Química de la Universidad de Sevilla.

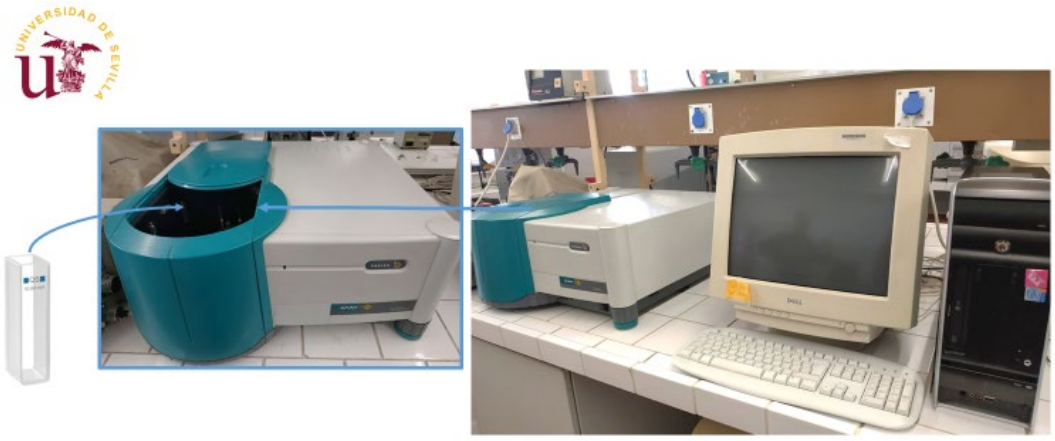


Figura 28. Imágenes del equipo de espectroscopía de fluorescencia y cubeta utilizados en el presente trabajo de tesis.

Las matrices de fluorescencia de emisión-excitación (EEM) fueron obtenidas variando el rango de longitud de onda de excitación (λex) entre 250 y 700 nm (cada 5 nm), y registrando la emisión (λem) de 300 a 800 nm (cada 2 nm). Para estas mediciones, las ranuras de excitación y emisión se ajustaron a 5 nm, y la velocidad de escaneo se fijó a 1200 nm min^{-1}. El sistema se calibró todos los días por medio de la longitud de onda del pico Raman de agua. Los espectros EEMs fueron registrados por triplicado para cada muestra y preprocesados para evitar áreas ruidosas y no informativas seleccionando rangos espectrales más cortos (λex de 250 a 680 nm, y λem de 310 a 800 nm).

3.2.1.3.2. Determinación de la presencia o concentración de caramelo de mosto

- Materiales y reactivos:
  - Caramelo de mosto (caramelo colorante MO-7) suministrada por SECNA S.A. (Valencia, España) con el número de identificación CEE: E-150d.
  - Agua de purificación Milli-Q (Millipore, EE. UU.)
  - Ácido acético calidad analítica de Merck (Darmstadt, Alemania)
  - Metanol de calidad analítica de Merck (Darmstadt, Alemania)
  - 5-Hydroxymethylfurfural (5-HMF) de Sigma-Aldrich (Madrid, España)
  - Espectrofotómetro de fluorescencia Varian Cary-Eclipse (Varian Iberica, Madrid, España) equipado con dos monocromadores Czerny-Turner, una lámpara de descarga pulsada de xenón a 80 Hz con altura media de pico de 2 µ (poder de pico equivalente a 75 kW) y un detector de tubo fotomultiplicador R298 de alto rendimiento.
  - Cubetas de cuarzo de 3,5 ml estándar (Hellma Analytics, Müllheim, Alemania) de 1 cm de longitud de trayectoria.
  - Recipiente termostatizado Peltier (25,00 ± 0,05 °C).
  - Ordenador con software Cary-Eclipse
  - Cromatógrafo de líquidos LaChrom® WWR-Hitachi (Barcelona, España) con una bomba cuaternaria L-7100 conectada a un detector de red de diodos L-7455 (DAD), acoplado a un automestreador L-2200.
  - Columna Luna C18, 5 µm, 250 × 4,6 mm y precolumna de protección de 4,0 × 3,0 mm de Analytical Phenomenex (Torrance, CA, EE. UU.).
  - Fase móvil de 80% de agua, 18% de metanol y 2% ácido acético.
  - Filtro de membrana 0.45 µm PTFE (Merck, Darmstadt, Alemania)

➢ Instrumentación y procedimiento analítico

Las muestras de vinagre de vino se analizaron directamente sin tratamiento previo de la muestra pipeteándolas en cubetas de cuarzo para su medición, usando el mismo método descrito en el apartado anterior, pero ajustando las longitudes de onda de excitación-emisión a 250 - 650 nm (cada 5 nm) y a 300 - 700 nm (cada 4 nm), respectivamente. Estos análisis se llevaron a cabo en el Departamento de Química Analítica de la Facultad de Química de la Universidad de Sevilla.

Se realizaron curvas de calibración de 13 puntos mediante la adición de diferentes cantidades de caramelo de mosto (entre 5 y 250 µL) a distintas matrices (vinagre de vino con DOP sin caramelo, vinagres de vino con DOP comerciales y matriz hidroacética 6%). Un esquema de estas curvas se muestra en la siguiente Figura 29.

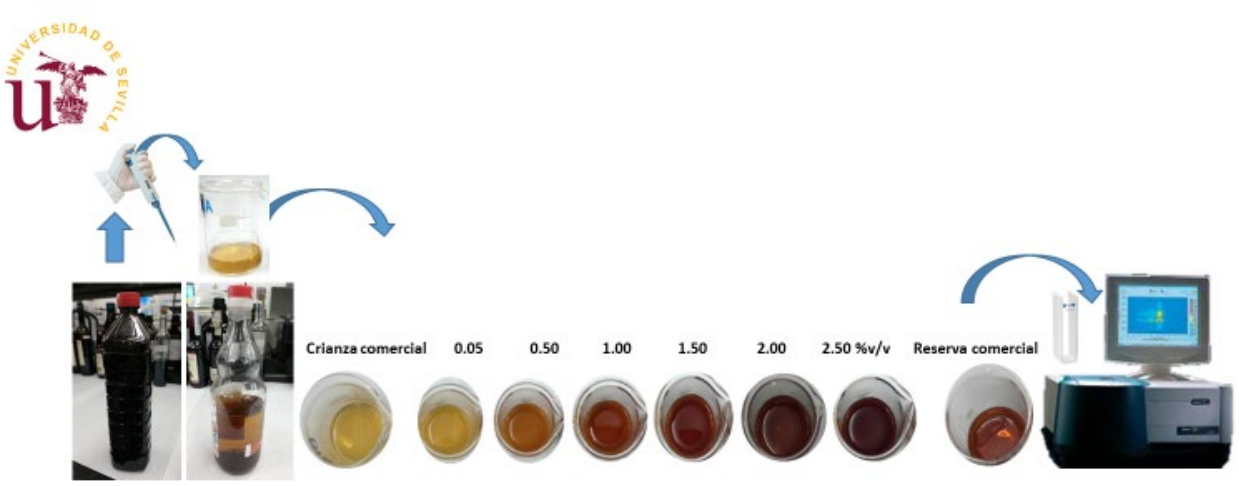


Figura 29. Esquema de la metodología utilizada para el análisis del caramelo de mosto por EFM. Se muestran las distintas adiciones de realizadas a una muestra de vinagre de vino Crianza, así como las botellas correspondientes al caramelo de mosto y vinagre de vino (izquierda) y el equipo de fluorescencia utilizado (derecha).

3.2.1.4. Resonancia magnética nuclear de protones (1H-RMN)

➢ Materiales y reactivos

- Sal sódica del ácido 3- (trimetilsilil) propiónico-2,2,3,3-d4 (TMSP-2,2,3,3-d4) de Merck (Darmstadf, Alemania)
- Óxido de deuterio, D_2O (99,96%), de VWR Chemicals (Leuven, Belgica).
- Espectrómetro Bruker AVIII 700 (Bruker Biospin GmbH Rheinstetten, Karlsruhe, Alemania)

- Tubos RMN DEU QUANT 5-7 de DEUTERO GMBH (Kastellaun, Alemania).
- Registro del espectro por secuencia de Bruker spin-echo “cpmgpr.fb” (Carr-Purcell – Meiboom – Gill, Bruker Library) con presaturación de agua
- Secuencia Bruker "baseopt" para optimización de línea base.
- Software Bruker TopSpin 3.0
- Chenomx NMR Suite 7.0 (Chenomx,Edmonton, Canada)

➢ Instrumentación y procedimiento analítico

Las muestras se prepararon mediante la adición de 100 µL de 0,16% de TMSP-2,2,3,3-d4 en disolución de D2O, a 600 µL de cada vinagre de vino. El TMSP se usó como referencia de cambio químico (δ = 0) y estándar interno. Los espectros de ^{1}H-RMN se adquirieron a 300 K de temperatura en un espectrómetro que opera a 700.25 MHz. Los FID se registraron como la suma de 64 exploraciones de 7,4 s, cada una cubriendo un ancho espectral de 11,0 ppm con 1 s entre cada exploración consecutiva. El equipo y materiales utilizados se muestran en la siguiente figura (Figura 30). Estos análisis se llevaron a cabo en el Servicio de RMN del Centro de Investigación, Tecnología e Innovación (CITIUS) de la Universidad de Sevilla.

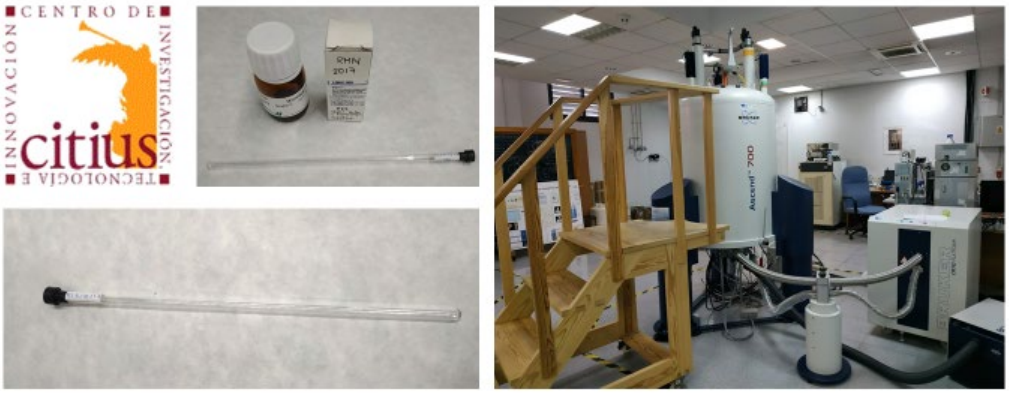


Figura 30. Material y equipo utilizado para el análisis de los vinagres de vino por ^{1}H-RMN.

3.2.1.5. Espectroscopía de Ultravioleta-visible (UV-vis)

➢ Materiales y reactivos

- Espectrofotómetro UV-vis CHEMUSB4 (USB4000) de Ocean Optics junto con un detector con matriz de diodos.
- Cubeta de cuarzo con una longitud de trayectoria de 10 mm (Hellma Analytics, Müllheim, Alemania)
- Agua ultrapura calidad Milli-Q (Millipore, EE. UU.)
- Ordenador con software OceanView

- Instrumentación y procedimiento analítico

Como el análisis directo de los vinagres de vino producía saturación de señal en el detector del espectrofotómetro, fue necesario un estudio de dilución previo, en el cual la dilución óptima seleccionada fue de 1/10 vinagre/agua (v / v), ya que permitía registrar el espectro de mayor intensidad, pero también la observación de las curvas espectrales de aquellos con la señal más baja. Las mediciones de espectroscopía UV-vis de estas muestras diluidas se realizaron utilizando un espectrofotómetro UV-vis CHEMUSB4 de Ocean Optics junto con un detector con matriz de diodos. Las muestras se colocaron en una cubeta de cuarzo y la absorbancia en función de la longitud de onda se midió con una resolución de 2 nm en un rango de trabajo de 180 a 890 nm, por duplicado. Se usó agua ultrapura (calidad MilliQ) como la exploración de referencia y se seleccionó el rango de 280 a 600 nm como la región con información relevante. Un esquema del procedimiento seguido, así como el material y equipo utilizado se muestra en la siguiente figura (Figura 31). Este equipo pertenece al centro de trabajo de la Dra. Silvana M. Azcárate, la Facultad de Ciencias Exactas y Naturales, Universidad Nacional de La Pampa (CONICET) e Instituto de Ciencias de la Tierra y Ambientales de La Pampa (INCITAP), de Santa Rosa, Argentina.

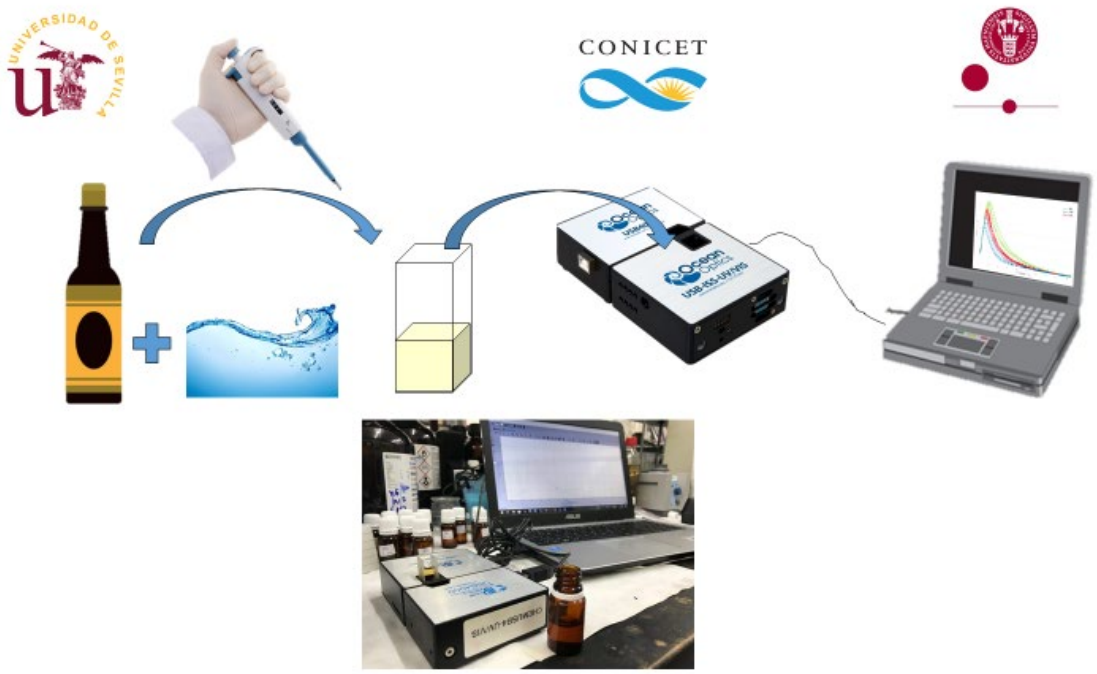


Figura 31. Procedimiento esquematizado, material y equipos utilizados para el análisis de los vinagres de vino por espectroscopía UV-vis.

3.2.2. ANÁLISIS CROMATOGRÁFICO: GC-MS, GC-MS-O

- Materiales y reactivos*:*
  - 4-methyl-2-pentanol (Merck, Darmstadf, Alemania)
  - Ethanol (Merck, Darmstadf, Alemania)
  - Agua ultrapura de calidad Milli-Q (Millipore, USA)
  - Serie de N-alcanos de cadena lineal C10 a C40 de Fluka (Madrid, España)
  - Barra de agitación larga de 10 mm recubierta con PDMS de la marca Twisters® (Gerstel, Müllheim and der Ruhr, Alemania)
  - Tubos sorbentes Tenax TA™ (Gerstel, Müllheim and der Ruhr, Alemania)
  - Fibra de SPME recubierta con 50/30 µm de divinilbenceno / Carboxen en fibra PDMS (DVB /Carboxen/PDMS) de Sigma-Aldrich (Bellefonte, PA, USA)
  - Estándares de compuestos volátiles utilizados en la identificación de Sigma-Aldrich (Madrid, España), Merck (Darmstadf, Alemania) y Fluka (Madrid, España).
  - Diclorometano de calidad analítica Merck (Darmstadt, Germany)
  - Sulfato de sodio anhidro de calidad analítica Merck (Darmstadt, Germany)
  - Cloruro de sodio de calidad analítica Merck (Darmstadt, Germany)
  - Ácido acético de calidad analítica Merck (Darmstadt, Germany)
  - Columna CPWax-57CB, con 50 m x 0,25 mm y 0,20 µm de espesor de película (Varian, Middelburg, Países Bajos)
  - Columna HP5 de 30.0 mx 0.25 mm y 0.25 lm de espesor de película (Agilent)
  - Sistema de Desorción Térmica (TDS2) conectada a un inyector con enfoque criogénico CIS-4PTV (Gerstel)
  - Unidad de Espacio en Cabeza Dinámico (Gerstel)
  - Sistema GC 6890 Agilent acoplado con un espectrómetro de masas de cuadrupolo simple Agilent 5975inert
  - Sistema GC Bruker 450 acoplado a un espectrómetro de masas de triple cuadrupolo Bruker 320.
  - Ordenador con software MS ChemStation (Agilent technologies Inc.) y Star Cromatography Workstation (Varian CA 94598-1675/USA)

- Instrumentación y procedimiento analítico

Las técnicas de extracción estudiadas para los distintos análisis cromatográficos de este presente trabajo de tesis se resumen a continuación y su procedimiento se muestra en la Figura 32.

3.2.2.1. Extracción por sorción en espacio en cabeza estático (HSSE)

Las condiciones de extracción y desorción óptimas para el análisis de las muestras de vinagre de vino por HSSE-GC-MS se realizaron de acuerdo al método previamente validado por (R. M. Callejón, González, Troncoso, & Morales, 2008). Este método será descrito en detalle en su correspondiente capítulo (Capítulo VI) de la presente memoria de tesis. Tras la extracción, la desorción de los Twister ® se realizó en el sistema GC 6890 Agilent acoplado con un espectrómetro de masas de cuadrupolo simple Agilent 5975inert (Agilent Technologies, Santa Clara, CA, USA) equipado con un sistema de Desorción Térmica (TDS2) conectado a un inyector con enfoque criogénico CIS-4PTV , perteneciente al grupo de investigación AGR167 de la Universidad de Sevilla (Figura 32).

3.2.2.2. Extracción por espacio de cabeza dinámico (DHS)

Las condiciones de extracción y desorción para el análisis de las muestras de vinagre de vino por DHS-GC-MS se realizaron usando la unidad de Gerstel de Espacio en Cabeza Dinámico, bajo las condiciones del método de (Ubeda et al., 2016). Este método será descrito en detalle en su correspondiente capítulo (Capítulo VI) de la presente memoria de tesis. Esta extracción se realizó en el sistema GC 6890 Agilent acoplado con un espectrómetro de masas de cuadrupolo simple Agilent 5975inert descrito en el apartado anterior, perteneciente al grupo de investigación AGR167 de la Universidad de Sevilla (Figura 32).

3.2.2.3. Microextracción en fase sólida en espacio de cabeza (HS-SPME)

El método utilizado para el análisis de las muestras por HS-SPME-GC-MS fue adaptado de los métodos previamente validados de (Cirlini, Caligiani, Palla, & Palla, 2011; Natera Marín, Castro Mejías, de Valme García Moreno, García Rowe, & García Barroso, 2002; Pizarro, Esteban-Díez, Sáenz-González, & González-Sáiz, 2008). Este método será descrito en detalle en su correspondiente capítulo (Capítulo VI) de la presente memoria de tesis. En este caso, esta extracción y desorción de la fibra se realizaron en el Sistema GC Bruker 450 acoplado a un espectrómetro de masas de triple cuadrupolo Bruker 320, perteneciente al Centro de Investigación Tecnológica e Innovación de la US (CITIUS) (Figura 32).

3.2.2.4. Extracción Líquido-Líquido (ELL)

La extracción utilizada para el análisis olfatométrico de las muestras de vinagre de vino (detallado en el Capítulo VII) se realizó mediante el método de extracción líquido-líquido (ELL), de acuerdo con la metodología validada previamente por Ferreira et al. (2003) (Silva Ferreira, Barbe, & Bertrand, 2003) y utilizado por los autores en trabajos anteriores (Raquel M Callejón, Morales, Troncoso, & Silva Ferreira, 2008) (Figura 32).

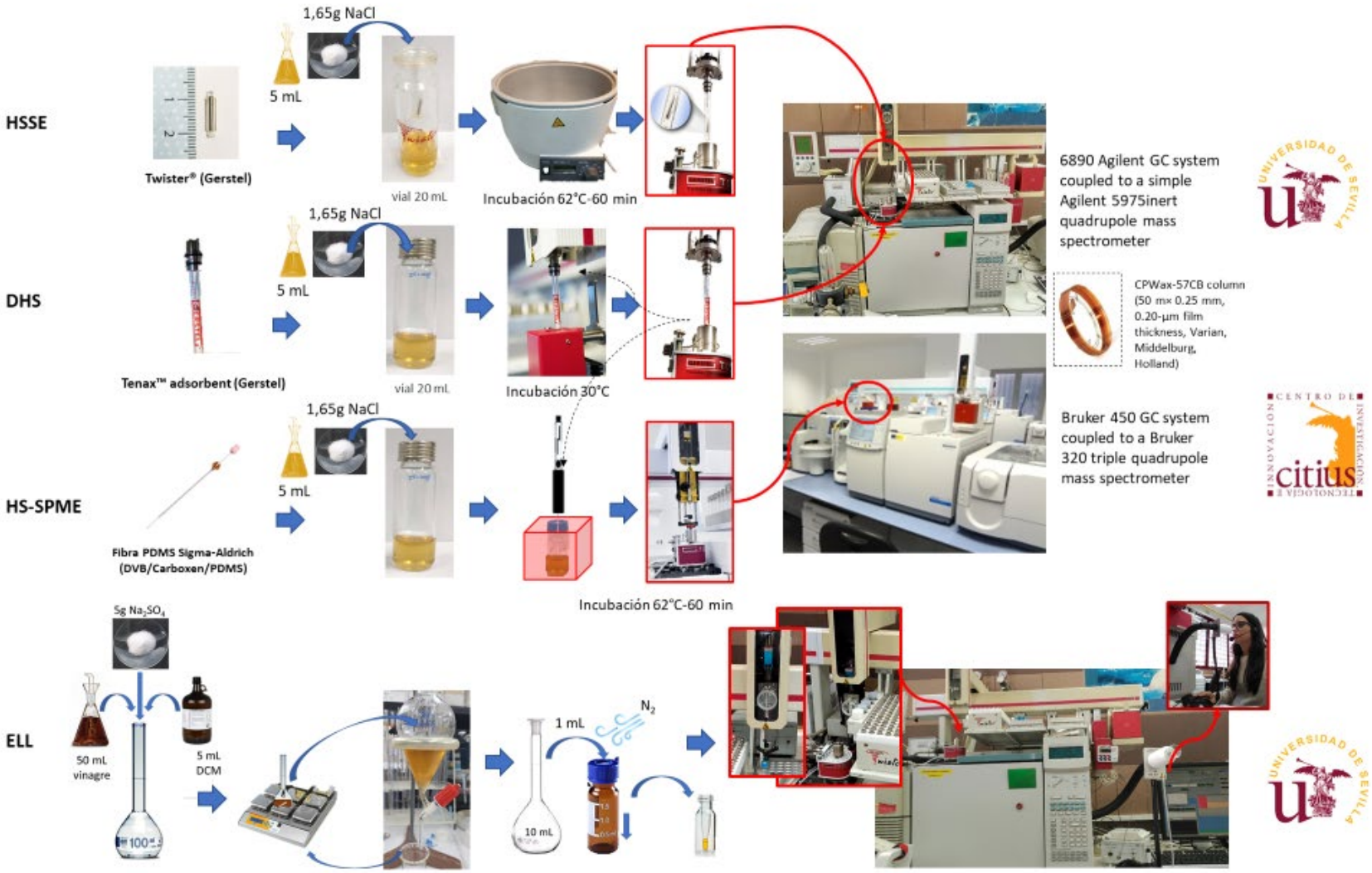


Figura 32. Esquema del procedimiento seguido en el análisis cromatográfico (GC-MS y GC-MS-O) por las diferentes técnicas de extracción

- Condiciones cromatográficas para el análisis por GC-MS:
    - Temperatura del horno a 35°C durante 5 minutos, aumentando hasta 220°C a 2.5°C/min y se mantuvo durante 5 min.
    - Temperatura de la línea de transferencia constante a 300°C.
    - Helio como gas portador a flujo de 1 mL/min.
- Condiciones cromatográficas para el análisis olfatométrico (GC-MS-O):
    - Inyección de 5 µl de la muestra extraída con el puerto del inyector calentado a 220 °C en modo sin división durante 1 minuto, con un flujo total de 73,5 ml/min.
    - Temperatura del horno de 40 °C (durante 1 min) aumentando a 2°C/min a 220°C manteniéndose durante 30 min.
    - Efluente de la columna dividido 2:3 en un detector de espectroscopia de masas (MS) y un puerto de aspiración calentado.
    - Temperatura del inyector y del detector de 250 °C.
    - Temperaturas de cuadrupolo, fuente y línea de transferencia a 150, 230 y 280 ° C, respectivamente.
- Condiciones y equipo de detección para ambos análisis:
    - Temperaturas de cuadrupolo, fuente y línea de transferencia mantenidos a 150, 230 y 280 ° C, respectivamente.
    - Espectros de masas de ionización de electrones en el modo de exploración completa registrados a 70 eV de energía electrónica en el rango de 35–350 m/z.

Además, para el análisis GC-MS-O, una vez inyectada la muestra, un panel de catadores realizó una serie de análisis por muestra, oliendo el efluente de la columna y dando una descripción verbal de cada olor percibido y su nivel de intensidad. Los resultados se expresaron como la "frecuencia modificada" (MF), que se calculó utilizando la fórmula MF (%) = [F (%) x I (%)] 1/2 propuesta por Dravnieks, (1985), en la que F es la frecuencia de aparición y la intensidad I (Dravnieks, 1985).

Las diferentes partes del equipo utilizado para los análisis cromatográficos realizados en nuestro departamento se muestran en la Figura 33.

Figura 33. Equipo de GC 6890 Agilent utilizado para el análisis cromatográfico (GC-MS y GC-O-MS) de las muestras de vinagre de vino. a) Estación de extracción o brazo automático de muestreo, b) Horno y espectrómetro de masas; c) ODP o nariz olfatométrica.

3.2.3. ANÁLISIS SENSORIAL

- Materiales y reactivos
  - *Copas opacas*
  - *Tapaderas*
  - *Discos identificadores*
  - *Fichas de cata*
- Instrumentación y procedimiento analítico

En el presente trabajo de tesis se realizaron diferentes análisis sensoriales dependiendo de la finalidad buscada. Así, se desarrolló una prueba sensorial para evaluar la influencia del caramelo de uva agregado en las propiedades organolépticas de los vinagres de vino con DOP para proponer un posible límite de adición que no afecte ni modifique sus propiedades organolépticas (Capítulo IV de la presente memoria de tesis). Por otro lado, se realizaron análisis sensoriales en conjunto con el análisis GC-MS-O para determinar la relación de los olores de impacto con los perfiles sensoriales, así como tratar de determinar qué atributos sensoriales que

marcan la diferencia y para determinar el umbral o límite de detección de ciertos compuestos o aromas de impacto (Capítulo VII de la presente memoria de tesis). En la Figura 34 se muestran imágenes de distintas sesiones de cata realizadas para el presente trabajo de tesis.

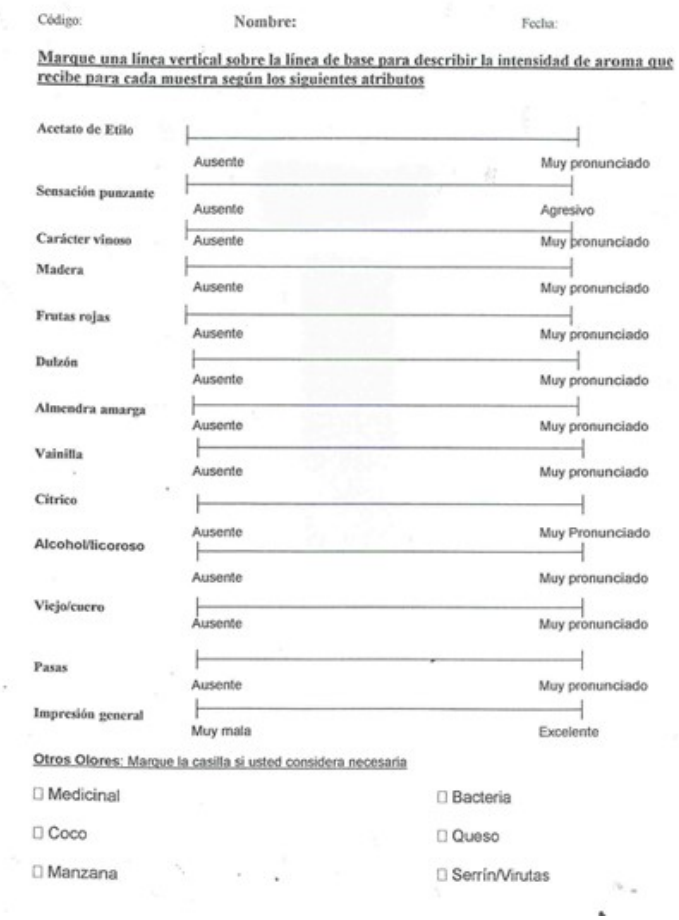
Figura 34. Imágenes de distintos análisis sensoriales realizados con diferentes fines.

El panel sensorial experto que llevó a cabo las diferentes pruebas descritas en este trabajo estuvo compuesto por ocho catadores (cinco mujeres y tres hombres), pertenecientes al Departamento de Nutrición y Bromatología de la Facultad de Farmacia de la Universidad de Sevilla y colaboradores externos de la Universidad de Módena y Reggio Emilia y el CONICET de Argentina. Todos contaban con experiencia en el análisis sensorial del vinagre de vino y fueron además previamente entrenados. La capacitación se realizó de acuerdo con los protocolos internacionales (ISO.4120: 1983; ISO.6658: 1985).

Entre las diferentes pruebas sensoriales realizadas en el presente trabajo de tesis, se han realizado las siguientes:

3.2.3.1. Pruebas descriptivas

Se realizó una prueba descriptiva para describir el aroma de los vinagres de vino mediante el protocolo establecido y validado por (Tesfaye et al., 2010) y el método aprobado por la Sociedad Americana para Pruebas y Materiales (ASTM). Esta prueba proporciona una descripción sensorial completa de los productos, cuantificando la intensidad percibida de la característica sensorial del producto. En él, los jueces describieron las muestras según la intensidad y la detección de los distintos descriptores mediante la cumplimentación de la siguiente ficha de cata (Figura 35).



Código: Nombre: Fecha:

Marque una línea vertical sobre la línea de base para describir la intensidad de aroma que recibe para cada muestra según los siguientes atributos

Acetato de Etilo	Ausente	Muy pronunciado
Sensación punzante	Ausente	Agresivo
Carácter vinoso	Ausente	Muy pronunciado
Madera	Ausente	Muy pronunciado
Frutas rojas	Ausente	Muy pronunciado
Dulzón	Ausente	Muy pronunciado
Almendra amarga	Ausente	Muy pronunciado
Vainilla	Ausente	Muy pronunciado
Cítrico	Ausente	Muy Pronunciado
Alcohol/licoroso	Ausente	Muy pronunciado
Viejo/cuero	Ausente	Muy pronunciado
Pasas	Ausente	Muy pronunciado
Impresión general	Muy mala	Excelente

Otros Olores: Marque la casilla si usted considera necesaria

☐ Medicinal ☐ Bacteria
☐ Coco ☐ Queso
☐ Manzana ☐ Serrín/Virutas

Figura 35. Ficha de cata usada en pruebas descriptivas.

3.2.3.2. Pruebas de ordenación

Estas pruebas se realizaron para determinar los umbrales de ciertos aromas, así como para el completar el estudio de la adición de caramelo de mosto a los vinagres de vino, siguiendo el protocolo para vinagres establecido por Tesfaye et al. (2010). Para ello, se preparan disoluciones correspondientes a cada uno de los atributos a entrenar en concentraciones crecientes, las cuales son presentadas de manera aleatoria al catador, el cual tiene que identificar el descriptor y ordenar las soluciones en orden creciente en función de la intensidad, constituyendo una escala ordinal según la ficha de cata que se muestra en la Figura 36.

Ordena de menor a mayor las muestras según la intensidad del olor:				
¿A qué te huele?				

Figura 36. Modelo de ficha de cata para pruebas de ordenación.

3.2.3.3. Pruebas triangulares

Estas pruebas se realizaron según el método descrito en la IS0 4120-1983, con el fin de determinar si los panelistas eran capaces de discriminar muestras sin caramelo de mosto de aquellas con adiciones, muestras envejecidas modificadas de las no modificadas, así como para la determinación de umbrales. Las pruebas triangulares son pruebas de diferencias en las que se presentan tres muestras marcadas en clave, dos de las cuales son idénticas. El juez deberá indicar cuál de las muestras es diferente. La ficha de cata utilizada se muestra en la Figura 37. Ésta constaba de escalas no estructuradas de 10 cm de longitud en las cuales los jueces marcaban con una X la intensidad de cada atributo.

NOMBRE: FECHA:

PRUEBA TRIANGULAR

Escribe en cada casilla el código de las muestras y rodea con un círculo la muestra que considera diferente. Señale qué atributos considera que marcan dicha diferencia y cual de las muestra considera con mayor calidad aromática.

	Clave (Izquierda)	Clave (Medio)	Clave (Derecha)
1			
¿Qué muestra es mejor?			
Atributo que consideras que marcan la diferencia:			

	Clave (Izquierda)	Clave (Medio)	Clave (Derecha)
2			
¿Qué muestra es mejor?			
Atributo que consideras que marcan la diferencia:			

	Clave (Izquierda)	Clave (Medio)	Clave (Derecha)
3			
¿Qué muestra es mejor?			
Atributo que consideras que marcan la diferencia:			

PRUEBA TRIANGULAR

Nº de Mesa:

Muestra: Vinagre (OLOR)		Nombre: Fecha: Hora:	
Examine las tres muestras. Rodee con un circulo la clave de la muestra que considera distinta.			
Juego Nº	Clave (1)	Clave (2)	Clave (3)
1			
2			
3			
4			
5			

Figura 37. Fichas de cata para pruebas triangulares.

3.2.4. ANÁLISIS ISOTÓPICO $^{13}C/^{12}C$ Y $^{18}O/^{16}O$

- Materiales y reactivos
  - Analizador elemental Carlo Erba 1108 acoplado en modo de flujo continuo a un IRMS (Espectrómetro de masas de relación isotopica) VG Isocrom.
  - Estándares NBS-22, OIEA CH6 y IAEA 600, V-SMOW2 y SLAP2.
  - GasBench acoplado en modo de flujo continuo a un espectrómetro de masas de relación de isótopos (IRMS) Delta V Advantage, Bremen (Alemania).

➢ Instrumentación y procedimiento analítico

Los resultados del ratio $^{13}C/^{12}C$ y $^{18}O/^{16}O$ se expresan en notación delta estándar (δ) según la desviación de mil (‰) de los estándares VPDB (Vienna-Pee Dee Belemnite, IAEA, Vienna) normalizados mediante la asignación de valores de consenso (Brand, Coplen, Vogl, Rosner, & Prohaska, 2014) y V-SMOW (Vienna-Standard Mean Ocean Water) normalizado a la escala VSMOW – SLAP (Prescripción Antártica Ligera Estándar), respectivamente. Los equipos utilizados se muestran en la Figura 38, siendo estos análisis realizados en el Laboratorio de isótopos estables de la Universidad Autónoma de Madrid y los métodos utilizados se describen a continuación:

Figura 38. Equipos utilizados para el análisis de isótopos estables de las muestras de vinagre de vino del presente trabajo.

- La determinación de $\delta^{13}C$ se realizó quemando la muestra a 1020 °C en un analizador elemental Carlo Erba 1108 acoplado en modo de flujo continuo a un IRMS (Espectrómetro de masas de relación isotopea) VG Isocrom. La precisión analítica, basada en el análisis repetido de las aguas estándar internas, fue de 0,1 ‰.
- La determinación de $\delta^{18}O$ se realizó mediante un proceso de equilibrio con una mezcla de gases He-CO2 durante 18 h y un análisis adicional de CO_2 en un GasBench acoplado, en modo de flujo continuo, a un espectrómetro de masas de relación de isótopos (IRMS). La precisión de medición en los estándares utilizados fue de 0.2 ‰.

3.2.5. ANÁLISIS QUIMIOMÉTRICO DE LOS DATOS

Para llevar a cabo el procesado de datos se ha utilizado el programa Excel como interfaz entre la información proporcionada por los instrumentos analíticos y los programas utilizados para el tratamiento de los datos, creación de modelos, etc.

Los programas o softwares de análisis de datos utilizados son los siguientes:

- Matlab 2015b (The Mathworks Inc., Natick, MA).
- PLS_Toolbox 7.9.5 y 8.2.1 (Eigenvector Research Inc., Wenatchee, WA)
- Infostat software (Grupo InfoStat, Argentina).

Con respecto a los diferentes algoritmos y procedimientos estadísticos usados para interpretar los resultados numéricos de acuerdo con los objetivos del trabajo, estos se muestran en la siguiente tabla (**Tabla 7**). Estas metodologías y sus abreviaturas correspondientes han sido descritas en la sección de Introducción.

Tabla 7. Algoritmos y metodologías utilizadas en los distintos capítulos que componen la presente memoria de tesis.

Algoritmos univariantes, de pretratamiento y normalización de datos	Algoritmos multivariantes	
Media	ANOVA	SIMCA
Desviación estándar	PCA	SVM
SNV	PARAFAC	HM
SMT	MCR	P-ComDim
Corrección línea base	PLS	
MC	N-PLS	
Scaling	PLS-DA	
Autoscaling	N-PLSDA	

4. RESULTADOS Y DISCUSIÓN

RESULTS AND DISCUSSION

BLOQUE I:

CARACTERIZACIÓN Y CLASIFICACIÓN ESPECTROSCÓPICA DE LOS VINAGRES DE VINO ESPAÑOLES CON DOP

CAPÍTULO I:
TÉCNICAS DE ESPECTROSCOPÍA VIBRACIONAL (ATR-FTIR Y NIR)

CHAPTER I.

Vibrational spectroscopic techniques (ATR-FTIR and NIR)

RESUMEN

Este capítulo se centra en el análisis de las muestras de vinagre de vino con DOP por técnicas de espectroscopía vibracional, en concreto, por espectroscopia de infrarrojo medio (MIR) y espectroscopía de infrarrojo cercano (NIR). Ambas técnicas fueron elegidas debido a sus altos potenciales como técnicas rápidas, económicas y no destructivas para la caracterización de vinagres de diferentes tipos. Sin embargo, ambas tienen una serie de ventajas y limitaciones, y por tanto se necesita evaluar y seleccionar la técnica espectroscópica más apropiada para la naturaleza de la muestra, el vinagre de vino en este caso. Así, en el presente capítulo se evaluaron las muestras de vinagre de vino con DOP por ATR-FTIR y NIR, comparándose los resultados de caracterización y clasificación obtenidos por ambas técnicas.

En primer lugar, el primer trabajo, publicado en Food Control 78 (2017) 230-237, presenta los resultados obtenidos del análisis de las muestras de vinagre de vino por espectroscopia de infrarrojo por Transformada de Fourier (FTIR) acoplada a un cristal de Reflactancia Total Atenuada (ATR). Para ello, 67 vinagres de vino con DOP pertenecientes a diferentes categorías y proporcionados por los Consejos Reguladores de cada DOP, fueron analizados: 36 “Vinagre de Jerez” y 31 “Vinagre Condado de Huelva”. La DOP “Vinagre de Montilla-Moriles” no fue incluida en este estudio ya que esta DOP se encontraba en su primer año de certificación y todavía no disponíamos de muestras de vinagre de vino en el año en el que se realizó el estudio. Aun así, posterior a esta publicación, las muestras de la DOP “Vinagre de Montilla-Moriles” fueron finalmente analizadas por la misma metodología, mostrando los mismos patrones que se observaron para las otras dos DOPs. Esta técnica permitió observar, casi directamente mediante la visualización del espectro, apoyándose en los resultados del análisis de componentes principales (PCA), diferencias entre las categorías establecidas en cada DOP, debidas a compuestos que durante el envejecimiento incrementan su concentración (como por ejemplo ácido acético, alcoholes y ésteres) o que son característicos de la categoría Pedro Ximénez (como por ejemplo azúcares y furfurales). La habilidad esta técnica para distinguir los vinagres por sus categorías se basó principalmente en una serie de bandas observadas en la región de 1500-900 cm^{-1}.

Este estudio permitió probar por primera vez diferencias entre las categorías de envejecimiento de las dos DOP de vinagres mediante un análisis directo, rápido y económico, demostrando la posible utilidad de la técnica ATR-FTIR para la caracterización de las categorías de envejecimiento establecidas de los vinagres de vino de alta calidad protegidos bajo una DOP. Este procedimiento además permitió caracterizar vinagres dulces (“Pedro Ximénez”) cuyo

espectro era claramente diferente, incluso a simple vista, en comparación con las categorías de envejecimiento, principalmente debido a las bandas asignadas a azúcares y compuestos de Maillard localizadas en las regiones del espectro de 1175 a 1000 cm^{-1}.

Las ventajas de ésta técnica (rápida, no destructiva y sin necesidad de preparación de muestra) podría permitir la implementación de este procedimiento como un control adicional para los Consejos Reguladores de las DOPs y productores para evaluar la autenticidad de cada categoría dentro de la DOP, y monitorizar el proceso de envejecimiento con un procedimiento simple y rápido.

Este primer artículo ha sido premiado con el "Premio a la Publicación Científica del mes de Farmacia, marzo de 2017".

Sin embargo, mediante este análisis no se consiguieron buenos resultados de clasificación de las tres DOP y sus categorías, por lo que, en la búsqueda de la técnica espectroscópica más apropiada para el análisis de estos vinagres, se planteó analizar las muestras de vinagre de vino con DOP mediante la espectroscopía de infrarrojo cercana (NIR), ya que esta técnica había demostrado ampliamente su utilidad en la clasificación y en la autenticación de alimentos. Los resultados obtenidos de este estudio se muestran en el segundo trabajo que se presenta en este capítulo, publicado en Food Control 89 (2018) 108-116.

En este caso se consiguió ampliar el número de muestras a analizar haciendo un total de 83 vinagres de vino de las DOP españolas (41 muestras de "Vinagre de Jerez", 29 de "Vinagre de Condado de Huelva" y 13 de "Vinagre de Montilla Moriles" de diferentes categorías) y 11 vinagres sin DOP de distintas regiones en los mercados locales y bodegas. Todas ellas se analizaron por espectroscopia NIR a 12000-4000 cm^{-1}. El análisis de componentes principales (PCA) se realizó para explorar los espectros y se utilizó el Análisis Discriminante de Mínimos Cuadrados Parciales (PLS-DA) para construir modelos con diferentes propósitos de clasificación: clasificar las diferentes categorías comercializadas (envejecidas y dulces) dentro de la misma DOP, como se había intentado con MIR, y, por otro lado, clasificar vinagres con DOP de aquellos sin la certificación DOP.

Los resultados de la exploración de los datos NIR mediante modelos PCAs mostraron que el envejecimiento y la protección bajo una DOP tenían un efecto relevante en distintas zonas del espectro. Así, las bandas de absorción más implicadas en el envejecimiento fueron de ~ 5200 a ~ 6500 cm^{-1}, siendo asociadas a la presencia de agua y compuestos aromáticos y fenólicos; además, la categoría dulce "Pedro Ximénez" mostró bandas características en la misma región (~ 5600 cm^{-1}) asociadas principalmente a los azúcares.

Este estudio proporcionó además una comparación entre los resultados de caracterización y clasificación de los vinagres de vino con DOP por ambas técnicas. De este modo, a partir de los resultados obtenidos se pudo concluir que MIR puede ser mejor técnica analítica para la caracterización de los vinagres de vino y sus categorías debido a que ofrece la ventaja de poder visualizar casi directamente las bandas que producen las diferencias entre muestras, así como permite una asignación química más fácil, mientras que en NIR las diferencias entre espectros no son fáciles de observar a simple vista, ni de interpretar, y se requiere un procesamiento más complejo de los datos. Por otro lado, la alta capacidad de predicción obtenida en el estudio NIR (> 90% de clasificación correcta) en comparación con MIR, demuestra la mejor utilidad de esta metodología para la autenticación y clasificación de los vinagres de DOP y sus categorías, así como su diferenciación de vinagres sin DOP. Por lo tanto, la selección de una de las técnicas como método para la caracterización y autentificación de los vinagres de vino con DOP dependerá del propósito que se busque, o incluso se puede contemplar la opción de combinar ambas técnicas para obtener mejores resultados.

Este segundo artículo ha sido también premiado con el “Premio a la Publicación Científica del mes de Farmacia, julio de 2018”.

ARTÍCULO 1

ATR-FTIR as a potential tool for controlling high quality vinegar categories

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ATR-FTIR as a potential tool for controlling high quality vinegar categories

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ABSTRACT

Characterization of wine vinegars qualified with a Protected Designation of Origin (PDO) is crucial to certify their quality and authenticity. Spectroscopic techniques as Fourier transform mid infrared spectroscopy (FTIR) with Attenuated Total Reflectance (ATR) has been applied to investigate its potential as a rapid, cost-effective and non-destructive tool for characterizing different categories of high-quality vinegars. Spectra from 67 wine vinegars belonging to the PDOs "Vinagre de Jerez" and "Vinagre Condado de Huelva", including their different established categories, were analyzed in the 4000–600 cm^{-1} infrared region. Changes associated to categories were observed in the region 1800–900 cm^{-1}. These changes were assigned to certain compounds that increase during aging (e.g. acetic acids, alcohols, esters) or are characteristic of Pedro Ximenez category (e.g. sugars, furfural). Principal component analysis carried out on the most relevant spectral features, revealed that aging of vinegars clearly affect the ATR-FTIR spectra obtained in each PDO.

1. Introduction

Vinegar is a product obtained by a double fermentation process (alcoholic and acetous fermentation or acetification) by using a wide variety of methods and different raw materials (wine, honey, cider, etc.). In the past, vinegar was considered as a secondary product in the family of fermented products and lacked of any recognized quality standard. Nowadays, vinegar is considered as a necessary product in households all over the world and many consumers regard it as high quality product.

Wine vinegar is the most commonly used vinegar in Mediterranean countries and Central Europe. It is the result of the conversion of sugars from grape juice into ethanol by yeast and the subsequent oxidation of the ethanol by acetic acid bacteria (Ordóñez, Callejón, Morales, & García-Parrilla, 2013). The wine vinegars available in the market differ in raw materials and production process. Concerning the latter vinegars can be produced by either a quick acetification system or a slow traditional process (De la Haba, Arias, Ramírez, López, & Sánchez, 2014). The time and type of aging (in different kinds of woods) are other sources of variability and they greatly affect the vinegar quality. Due to these processes, chemical modifications related with aging and with the microbiological activity occur and they provide specific and singular properties to the final product, being highly appreciated by consumers (Marrufo-Curtido et al., 2012).

Some wine vinegars are traditionally linked to a certain geographical area, and they are protected by the European Union with a legal framework that provides the category of "Protected Designation of Origin" (PDO). A product with a PDO registration means that it is produced, processed and prepared in a given geographical area using a recognized know-how (Council Regulation (EC) 510/2006). Consequently, a PDO registration provides an additional protection of consumers against falsifications and it guarantees some specifications related to their chemical and sensory features (Chinnici et al., 2009).

The production of wine vinegar in Spain is centered in Andalusia (Southern Spain). Andalusia is a region traditionally associated to wine culture where wine vinegars have been protected by three different PDOs because of their unique characteristics: "Vinagre de

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Jerez" (also known as "Sherry wine vinegar"), "Vinagre Condado de Huelva" and recently "Vinagre Montilla-Moriles". The first two PDOs are already well established and widely commercialized whereas "Vinagre Montilla-Moriles" was just granted its PDO status in 2015 (EU 2015/48) and their vinegars are starting to appear on the market. Furthermore, within each PDO, there are different categories according to their aging time and type in wood barrels. The high quality of these PDOs are the consequence of the raw material (e.g. grape variety, origin), the wooden cask used (e.g. American Oak casks) and the methods of aging. Aging procedures in these PDOs comprises the "criaderas y solera" (also called dynamic aging system) and "añada" (also called static system) systems. The vinegar in the first system is aged in different butts in which aged and young vinegars are sequentially mixed, while the vinegar in the second system is aged in a single butt without mixing with other vinegars.

The regulation on PDO "Vinagre de Jerez" (BOJA, 2008a) describe three categories according to aging time in oak barrels by the dynamic system "criaderas y solera": "Vinagre de Jerez" (aged in wood at least 6 months),"Vinagre de Jerez Reserva" (with a minimum aging time of 2 years.), "Vinagre de Jerez Gran Reserva" (aged for 10 or more years). This regulation also describes semi-sweet categories (i.e. "Pedro Ximenez", "Moscatel").

The regulation on PDO "Vinagre Condado de Huelva" (BOJA, 2008b) also establishes the following categories: "Vinagre Condado de Huelva" (no aging), "Vinagre Viejo Condado de Huelva Solera" (aged at least 6 months), "Vinagre Viejo Condado de Huelva Reserva" (aged at least 2 years), all aged by the system "criaderas y solera". Furthermore, there is one more category named "Vinagre Viejo Condado de Huelva Añada" aged at least 3 years but in static aging system.

These vinegars have high prices in the market due to their high quality, the long aging time and hence, the high cost of their production. That explains that these products are vulnerable to fraud (Callejón et al., 2012; Sáiz-Abajo, González-Sáiz, & Pizarro, 2004) and new tools are required to fight against falsification or mislabeling. Furthermore, the growing consumer demand and the increasing diversity of wine vinegars have raised a need to characterize them and to provide an adequate quality control to defend their identity (Cerezo et al., 2008; Liu, He, & Wang, 2008; Marrufo-Curtido et al., 2012). Different sensory and physicochemical techniques such as gas-chromatography-mass spectrometry (GC-MS), atomic absorption spectrometry or high-performance liquid chromatography (HPLC) have been used to characterize vinegars (Cirlini, Caligiani, Palla, & Palla, 2011; Natera, Castro, De Valme García-Moreno, Hernández, & García-Barroso, 2003). However, these techniques are often expensive and time-consuming. Rapid methods based on non-targeted technique can provide a solution for food authentication (Baeten & Dardenne, 2002). In particular, Fourier transform infrared spectroscopy (FTIR) has become an important tool for quantitative analysis (Rodriguez-Saona & Allendorf, 2011). FTIR combined with chemometrics has gained wide acceptance in the identification of chemical compounds in foods for authenticity and classification purposes (Grassi, Amigo, Lyndgaard, Foschino, & Casiraghi, 2014; Moros, Iñón, Garrigues, & de la Guardia, 2008; Regmi, Palma, & Barroso, 2012; Tay, Singh, Krishnan, & Gore, 2002; Van de Voort, Ghetler, García-González, & Li, 2008).

The use of an accessory of Attenuated Total Reflectance (ATR) in FTIR studies allows the direct analysis of liquids in a simple and non-destructive manner with enough sensitivity (Gouvinhas, de Almeida, Carvalho, Machado, & Barros, 2015; Versari, Parpinello, Chinnici, & Meglioli, 2011). Thus, ATR-FTIR has proven to be an appropriate method for the authentication of several liquid foods such as vinegar (Dong, Zheng, Jiao, Lang, & Zhao, 2016; Guerrero, Mejías, Marín, Lovillo, & Barroso, 2010), olive oil, wine, milk or honey (Bendini et al., 2007; Bevin, Fergusson, Perry, Janik, & Cozzolino, 2006; Gouvinhas et al., 2015; Kelly, Petisco, & Downey, 2006; Tarantilis, Troianou, Pappas, Kotseridis, & Polissiou, 2008; Tena, Aparicio-Ruiz, & García-González, 2014). Scarce studies have been carried out in the characterization of vinegars belonging to PDOs and with different aging times, although some studies already pointed out the utility of different spectroscopic techniques (e.g. near infrared and fluorescence spectroscopy) in vinegar studies (De la Haba, Arias, Ramírez, López, & Sánchez, 2014; Callejón et al., 2012).

The aim of this work was to study the potential of ATR-FTIR for the characterization of wine vinegar categories (aged and sweet) established in two Andalusian PDOs ("Vinagre de Jerez" and "Vinagre Condado de Huelva"). For this purpose, the most relevant spectral bands were identified and the information that they provided were assessed in terms of chemical assignment and vinegar characteristics. Principal component analysis (PCA) was used as unsupervised method in order to explore and compare the data structure and to help in the interpretation of ATR-FTIR analysis.

2. Materials and methods

2.1. Samples

Sixty-seven wine vinegar samples belonging to different categories registered as Protected Designation of Origin, named "Vinagre de Jerez" and "Vinagre Condado de Huelva", were collected from different wineries of Andalusia (Spain). Samples were divided as follows: 36 "Vinagre de Jerez" and 31 "Vinagre Condado de Huelva" including samples of each established category (Table 1).

2.2. Chemicals

Acetic acid was obtained from Merck (Madrid, Spain), and it was of analytical quality. Water was obtained from a Milli-Q purification system (Millipore, USA).

2.3. ATR-FTIR spectroscopy analysis

Spectral data were collected on a Bruker Vertex 70 FTIR spectrometer equipped with a DGTS detector (Bruker Optics, Ettlingen, Germany). The sampling station was equipped with an overhead and detachable multi-reflection attenuated total reflectance accessory (ATR, six bounces, Specac, Orpington, U.K.). This accessory consists on a 45° angle ZnSe crystal mounted in a shallow channel for the sample. Each spectrum was recorded at the same temperature (22 °C) in the region of 4000–600 cm^{-1} by an average of 50 scans at a resolution of 4 cm^{-1}. Before scanning each sample, a background spectrum was taken with an empty ATR crystal and recorded in the computer. Each sample (250 μL) was spread uniformly through the ATR crystal using a micropipette. After the analysis, the ATR crystal was thoroughly cleaned to eliminate the presence of vinegar residues between measurements and then wiped with cotton. Spectra were examined using OPUS version 7.0 (Bruker Optics, Ettlingen, Germany) and manipulated with OMNIC software. Each sample was analyzed in triplicate.

2.4. Data analysis

Data processing and statistical analysis was performed with MATLAB version 8.4.0.150421 (R2014b) and PLS-toolbox version 7.0.2 (Eigenvector Research Inc., Manson, WA). Different pre-processing methods were studied prior to PCA including standard

Table 1
Wine vinegar samples analyzed in this study.

Protected Designation of Origin (PDO)	Categories	code	Aging time (months)	n
"Vinagre de Jerez"	"Vinagre de Jerez" or "Crianza"	JCR	≥6	15
	"Reserva"	JRE	≥24	15
	"Gran Reserva"	JGR	≥120	3
	"Pedro Ximenez"	JPX	–	3
"Vinagre Condado de Huelva"	"Condado de Huelva"	CSC	0	10
	"Viejo Solera"	CSO	≥6	7
	"Viejo Reserva"	CRE	≥24	10
	"Viejo Añada"	CAN	≥36	4

normal variate (SNV), first and second derivative. Moreover, the spectra were properly normalized by mean centering after preprocessing. After some trials and computations, preprocessing was found not to be necessary. Therefore, the raw spectra were just normalized by mean centering. Principal component analysis (PCA) was applied to the 1500–900 cm^{-1} spectral region of the total of samples in triplicate in order to explore the data structure and to identify the main sources of variability in the spectra. Full cross validation (leave-one sample-out) was applied, understanding as sample all the analytical replicates of the same sample. Factor loadings for each principal component were used to assess about the relative importance of each wavenumber and the correlation of them with specific compositional properties according to their category.

3. Results and discussion

3.1. Interpretation of ATR-FTIR wine vinegar spectra

The first step in this work was to identify the most remarkable bands related to the major compounds that characterize wine vinegar samples. Fig. 1 shows the ATR-FTIR spectra of some analyzed vinegars. For a comparative purpose, a spectrum of Millipore Q-purified water is shown, as well as a spectrum of a mixture of pure acetic acid and water. In the first one (Fig. 1, spectrum A; Table 2), the intense bands detected in the regions of 3800–2790 cm^{-1} and 1685–1550 cm^{-1} were mainly assigned to –OH groups of water. The ATR-FTIR spectra of a standard of acetic acid diluted in water (Fig. 1, spectrum B) showed the presence of three new bands: one at ~1711 cm^{-1} assigned to the C=O group of acetic acid, and two bands near ~1410 and ~1290 cm^{-1} explained by C–O stretching and C–O–H in-plane bending respectively (Moros et al., 2008; Silva, Feliciano, Boas, & Bronze, 2014; Tarantilis et al., 2008; Versari et al., 2011). Acetic acid also absorbs at 3800–2790 cm^{-1} due to C–H and O–H stretching, although it is not observable in the spectrum for being overlapped by O–H absorption of water (Moros et al., 2008). Concerning the spectra of vinegar samples (spectra C, D and E in Fig. 1), all the bands associated to water and acetic acid were the most intense ones. Other minor bands were characteristics of some specific kinds of vinegars

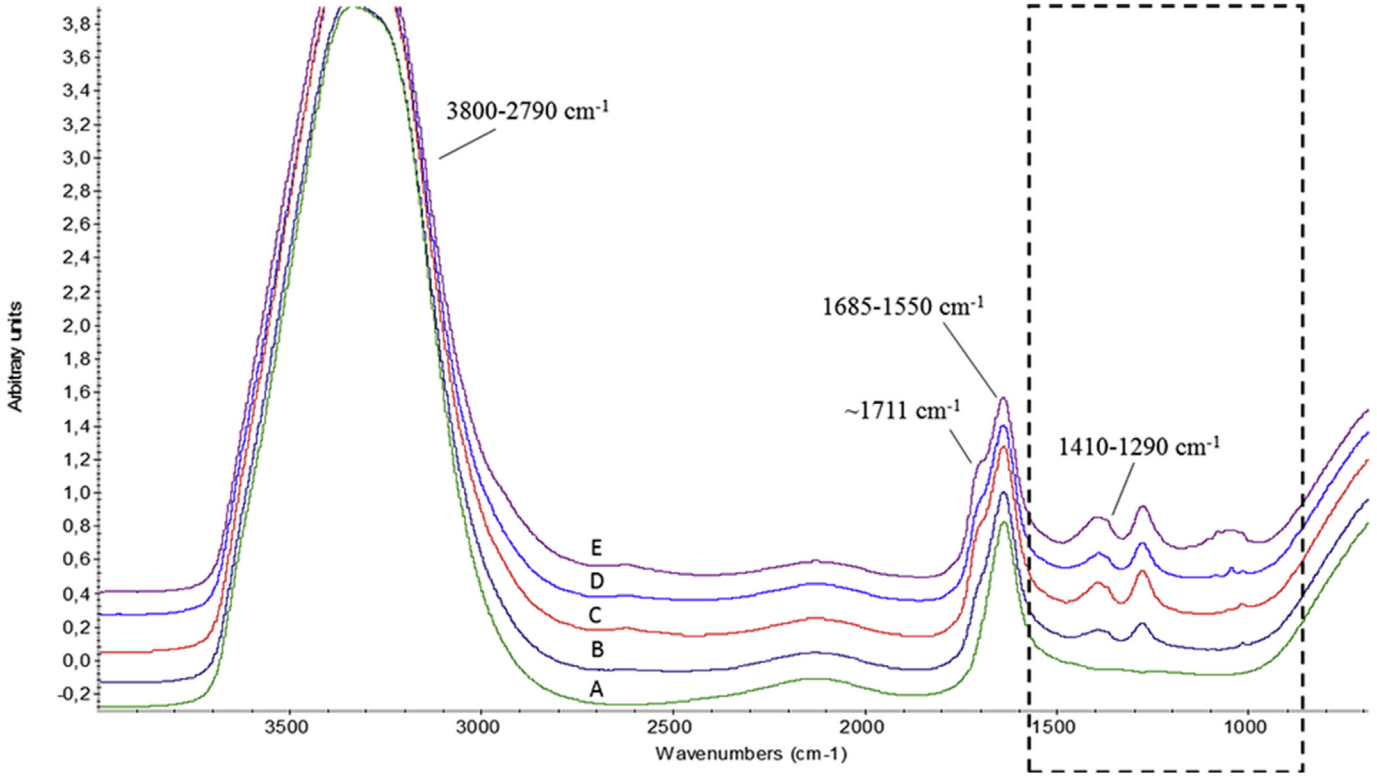

Fig. 1. Comparison between ATR-FTIR spectra of Millipore Q-purified water (A), a pure acetic acid solution in water (B), and some vinegar samples ("Vinagre de Jerez" PDO) of different categories: "Crianza" (C), "Reserva" (D) and "Pedro Ximenez" (E).

Table 2
Chemical assignment of the bands observed in the ATR-FTIR spectra of wine vinegars (Stuart, 2004; Aldrich library; Sigma Sample library).

Compounds	Principal Group Identified	Spectral Region (cm^{-1})
Water	O–H band	3800–2790; 1685–1550
Acetic acid	C–H, C=O, C–O stretching and C–O–H in-plane bending	3800–2790; 1800–1680; 1475–1230
Acids	C–O stretching	1300–1000
Ethanol, Glycerol	C–O stretching	1100–1000
Alcohols (e.g. 1-hexanol, 2-methyl-1-butanol)	O–H stretching band, C–O stretching	3600; 1575–900
Esters (e.g. ethyl acetate, isoamyl acetate, ethyl propanoate)	Aliphatic and aromatic C–O and C=O stretching	1700–1100
Sugars (fructose, glucose)	O–H, $-CH_2$	1065–1030
Aldehydes (e.g. Benzaldehyde)	C=O stretching	1700–1600
Phenols (e.g. guaiacol, eugenol, 4-ethylphenol)	–C–O, O–H stretching	1800–900
Furfurals	C=O, C=C	1300–840 and ~1020

(Table 2). Thus, the region at 1500–900 cm^{-1} also showed spectral bands assigned to complex interacting vibrations, resulting in a unique fingerprint for each vinegar.

Fig. 2 shows two ATR-FTIR spectra corresponding to an aged wine vinegar (Reserva category) and a "Pedro Ximenez" wine vinegar, both of them from PDO "Vinagre de Jerez". In addition to the bands assigned to water and acetic acid (spectral bands 1 to 6), the region 1500–900 cm^{-1} shows other absorption bands assigned to C–O, C–C, C–H, C–N, N–H and C=O groups (Stuart, 2004) (Table 2). These bands were produced by the presence of carboxylic acids, aldehydes, esters, ethers, alcohols and phenols and some nitrogen compounds presented in wine vinegar (Callejón et al., 2008b; Stuart, 2004). All these chemical groups have been identified in vinegars and they have an impact on quality properties (Callejón, Morales, Silva Ferreira, & Troncoso, 2008a). Fig. 2.A and 2.B shows the average spectra (region 1160–900 cm^{-1}) of the aged categories for "Vinagre de Jerez" and "Vinagre Condado de Huelva" respectively. Peaks 7, 8 and 9 (~1085, ~1045 and 1015 cm^{-1}) were assigned to alcohol compounds, aldehydes, and some esters and ethers as well as acids (Duarte, Barros, Almeida, Spraul, & Gil, 2004; Nieuwoudt, Prior, Pretorius, Manley, & Bauer, 2004; Versari et al., 2011). These bands were related to the aforementioned chemical compounds whose concentration increase during aging (Morales, Tesfaye, García-Parrilla, Casas, & Troncoso, 2001; Morales, Tesfaye, García-Parrilla, Casas, & Troncoso, 2002; Tesfaye, Morales, García-Parrilla, & Troncoso, 2002) being responsible for the variations in the absorbance intensities that are shown in the region 1160–900 cm^{-1} (Fig. 2.A and 2.B). Vinegars with longer aging period, such as "Vinagre de Jerez Gran Reserva" (JGR), "Vinagre de Jerez Reserva" (JRE), "Vinagre Condado de Huelva Reserva" (CRE), and "Vinagre Condado de Huelva Añada" (CAN), showed an increase in the intensity of the band at ~1045 cm^{-1} (band 8) and a slight increment at ~1085 cm^{-1} (band 7). These two spectral bands were explained by the presence of alcoholic and ester compounds (mainly ethanol and ethyl acetate), whose functional groups (C–O) absorb at this range of the spectrum (Duarte

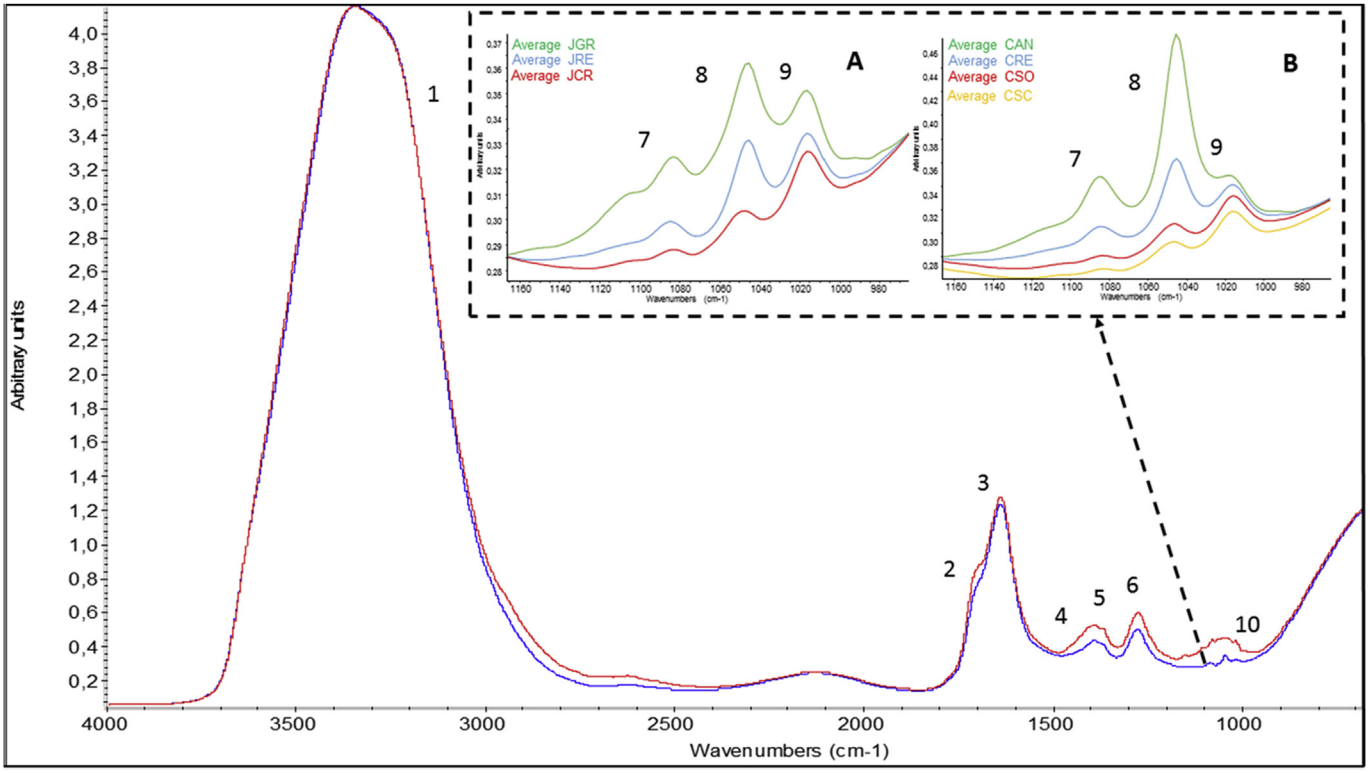

Fig. 2. ATR-FTIR spectrum of an aged wine vinegar ("Vinagre de Jerez Reserva") and a sweet vinegar ("Vinagre de Jerez Pedro Ximenez"). Two inserts (A and B) shows the selected spectral range of each PDO aged categories ("Vinagre de Jerez" and "Vinagre Condado de Huelva"). Note: (1) 3800–2790 cm^{-1}; (2) 1800–1680 cm^{-1}; (3) 1685–1550 cm^{-1}; (4) (5) (6) 1475–1230 cm^{-1} (7) ~1085 cm^{-1}; (8) ~1045 cm^{-1}; (9) ~1015 cm^{-1}; (10) 1175–1000 cm^{-1}. The acronyms for the different vinegar categories are defined in Table 1.

et al., 2004; Stuart, 2004). The spectral differences observed in aged categories agree with the fact that aging promotes several phenomena that affects the chemical composition of vinegars (Callejón, Morales, Silva Ferreira, & Troncoso, 2008b). The main changes involve water losses through wood pores, increases in acetic acid concentration, extraction of phenolic compounds from wood and formation of aroma compounds, mainly esters (Callejón et al., 2008b; García-Parrilla, Heredia, & Troncoso, 1999).

Fig. 2 also showed the spectra of a "Pedro Ximenez" vinegar, which is a particular sweet category of PDO "Vinagre de Jerez". Its spectrum showed a singular group of peaks in the region of 1175–1000 cm^{-1} (band 10, Fig. 2). This kind of vinegar has a high carbohydrate content and they suffer a Maillard reaction that induces the condensation of the reducing carbohydrates (glucose and fructose) and free amino acids producing an appearance of brown pigments and volatile compounds (Casale, Sáiz Abajo, González Sáiz, Pizarro, & Forina, 2006). Their characteristic bands in the region of 1175–1000 cm^{-1} mainly resulted from grape sugars and furfural compounds that are characteristics of these sweet vinegars (Casale et al., 2006). In fact, in previous studies the spectra of glucose and fructose showed a major peak at 1034 and 1062 cm^{-1} respectively (Sivakesava & Irudayaraj, 2000) and furfural at 1020 cm^{-1} and 1300–840 cm^{-1} (Dong et al., 2016) (Table 2).

3.2. Principal component analysis

In order to check the ability of the aforementioned bands to characterize Andalusian PDO vinegars and to find out if the spectral profile of these wine vinegars may be consistently correlated with specific compositional properties and/or sample aging, principal component analysis (PCA) was performed. PCA was used to detect groups of samples, outliers and to provide a visual representation of the relationships within the samples and between them (scores) and the variables (loadings). PCA was carried out with the spectral region 1500–900 cm^{-1}, previously preprocessed by mean centering, of the total of samples by triplicate from each PDO.

3.2.1. PDO "Vinagre de Jerez"

The two first principal components (PCs) of the different aged categories and Pedro Ximenez vinegars included in the "Vinagre de Jerez" PDO explained 97.33% of the total variance. Fig. 3 shows the scores and loadings plots in the plane defined by PC1 and PC2. PC1 allowed the separation of the "Pedro Ximenez" samples. Additionally, samples were distributed along PC1 according to their aging (Fig. 3-A): "Gran Reserva" with positive values of PC1; "Crianza", with negative values of PC1; and "Reserva" with both negative and positive values of PC1. This difference is mainly due to the wide range of aging time in these vinegars (from 6 months to >10 years). Nonetheless, some overlapping was observed in the PCA scores plot since aging is a factor that develops with time. Thus, "Reserva" vinegars (2–10 years) that were aged for slightly more than 2 years were observed close to "Crianza" category (6 months–2 years). Similar results were obtained in a previous work (Callejón et al., 2012) in which vinegars of different aging were analyzed by fluorescence spectroscopy. The loadings plot corresponding to the two PCs with higher amount of total variance, PC1 and PC2 (Fig. 3.B), shows that two specific regions of the spectra had more importance in the model (1175–1000 cm^{-1} and 1475–1230 cm^{-1}). PC1 (87.96% of the total variance) was mainly associated with positive values to the spectral region of 1175–1000 cm^{-1}. This region was ascribed to the samples with higher amount of sugars from grape-juice in their composition that was characteristic of "Pedro Ximenez" vinegars. These loadings for PC1 explained that Pedro Ximenez samples were separated from the rest of vinegars. In addition to this region, PC2 loadings showed positive values at the absorption bands characteristics of acetic acid (1475–1230 cm^{-1}) explaining that the majority of wine vinegar samples with more aging time were located in the positive side of PC2 (e.g. all "Gran Reserva" and many "Reserva" samples).

Once the Pedro Ximenez samples were explained and showed distinctive spectral characteristics, they were removed from the data in order to carry out a differentiation of the rest of vinegars (aged categories). Scores and loadings of the first two PCs are shown in Fig. 3.C and 3.D, accounting for 95.98% of the total variability. Once again, two regions of the spectra where highlighted in the loadings plot: 1475–1230 cm^{-1} and the two peaks at ~1085 cm^{-1} and ~1045 cm^{-1}. The highest contributions corresponded to the bands assigned to acetic acid and alcoholic, ethers and esters compounds respectively, indicating the relationship previously described (Fig. 2) between aging time and the increase of these compounds. The projection onto the reduced space spanned by the PCs allowed the separation of "Gran Reserva" and "Crianza" categories (with positive and negative values for PC1 respectively) in the score plot (Fig. 3.C), while the samples of the "Reserva" category were distributed between the two other groups, showing the importance of the aging in the spectral characteristics.

3.2.2. PDO "Vinagre Condado de Huelva"

A PCA was carried out with samples of all the categories of wine vinegar commercialized by "Vinagre Condado de Huelva" PDO (Fig. 4). The results obtained were similar to the PCA carried out for "Vinagre de Jerez" samples. Fig. 4 shows the scores and loadings plots of the first two principal components (PCs), accounting for 97.08% of the total variability. The score plot (Fig. 4.A) shows that PC1 explained most of the variability (81.39%) and allowed the differentiation between two groups: the less aged vinegars ("Vinagre Condado de Huelva") placed in the negative side of PC1, and the most aged vinegars ("Añada" and "Reserva") placed in the positive side. Moreover, from a conceptual standpoint, it is possible to rather affirm that the aging direction is not only PC1 but it is the diagonal of the PC1 vs PC2 plot, so that more aged vinegars result in a simultaneous increase in the scores along both components. Once again, there was an overlapping of samples belonging to the intermediate category "Solera", aged from 6 months to 2 years, with the category immediately more aged "Reserva", aged for more than 2 years. In regards to the loadings (Fig. 4.B), the highest contributions of PC1 corresponded to the bands assigned to acetic acid (1475–1230 cm^{-1}), and the positive loadings of PC1 and PC2 were assigned to alcoholic, ethers and esters compounds (1085 cm^{-1} and 1045 cm^{-1}). These highlighted regions confirmed again the relationship between aging time and the intensity of these bands. The loading profile shown in Fig. 4-B were similar to those obtained with the samples from "Vinagre de Jerez" PDO (Fig. 3-D).

These results suggested that FTIR spectra could be used to identify the main differences between the established categories of wine vinegars within each PDO. The "fingerprint" region of vinegar spectra has demonstrated to provide important information for characterizing each wine vinegar.

In addition to the differentiation of categories within each PDO, the spectral signals of the two PDOs were included in the same PCA model. In this study, only the categories with the same range of aging (>6 months and >24 months) were considered since they are comparable between PDOs (Table 1). Fig. 5.A shows the scores plot of the first two PCs, accounting for 93.98% of the total variability. The scores showed a clear differentiation between the two PDOs. These results reinforced that wine vinegars produced under the specifications of PDOs have unique quality and characteristics. The spectral loadings responsible for differentiation (Fig. 5.B) were again located in the ranges 1475–1230 cm^{-1} and 1175–1000 cm^{-1}, already selected in previous PCA models.

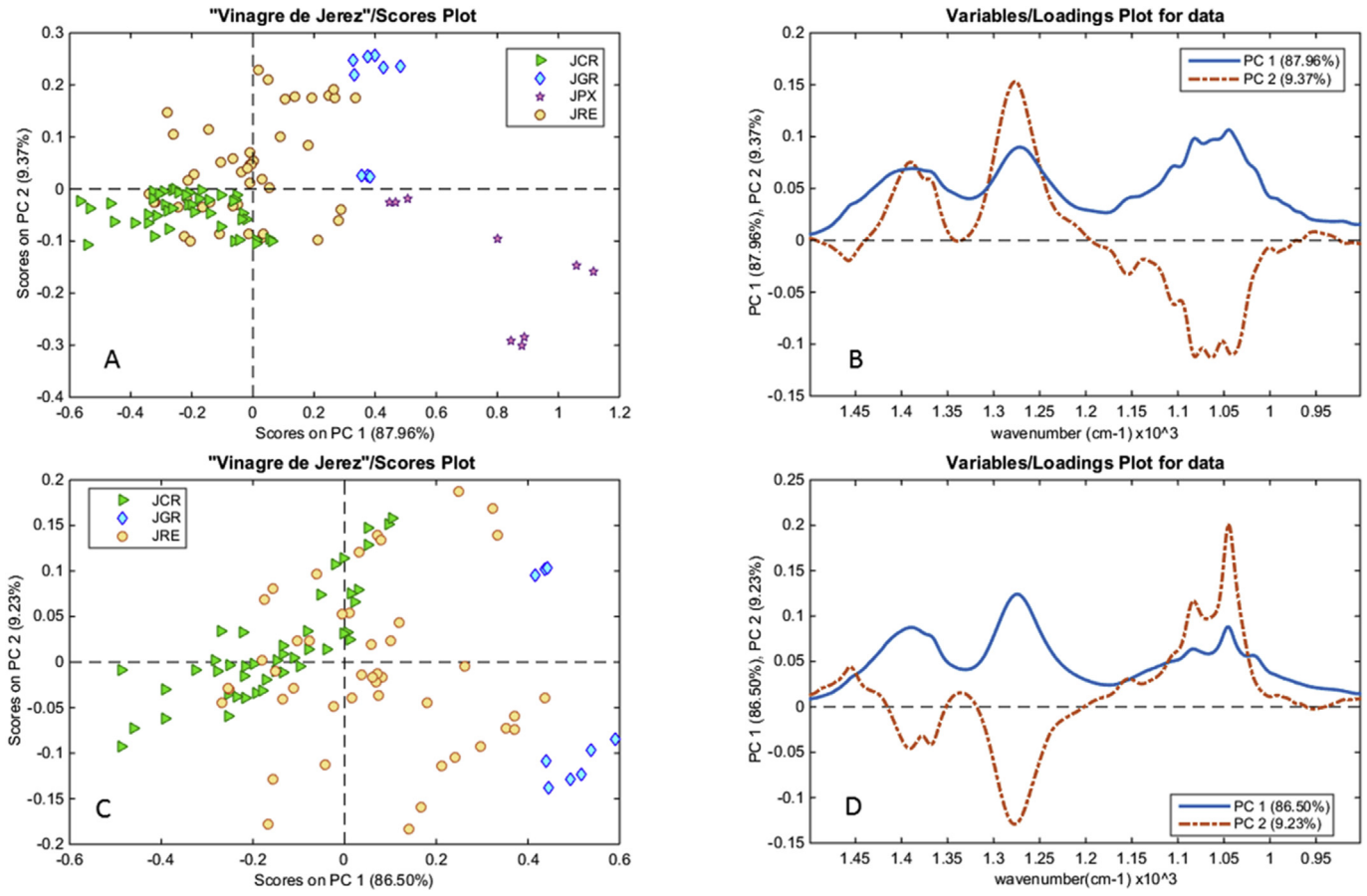

Fig. 3. Results of principal component analysis carried out with ATR-FTIR spectral data of "Vinagre de Jerez" samples (in triplicate). The score plots with and without "Pedro Ximenez" category (A and C respectively) are shown as well as the corresponding loadings of the first two principal components (PC1 and PC2) (B and D). The acronyms for the different vinegar categories are defined in Table 1.

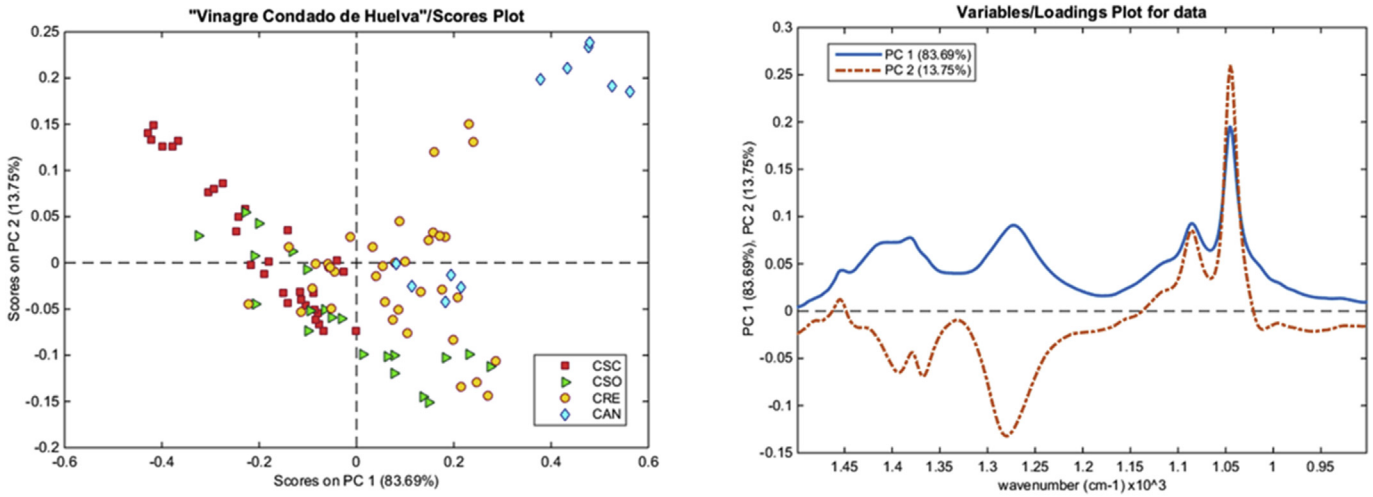

Fig. 4. Results of principal component analysis carried out with ATR-FTIR spectral data of "Vinagre Condado de Huelva" samples (in triplicate). The score plot (A) is shown as well as the corresponding loadings of the first two principal components (PC1 and PC2) (B). The acronyms for the different vinegar categories are defined in Table 1.

4. Conclusions

In this study, FTIR proved to be useful for a simple characterization of the established aging categories of high quality wine vinegars protected under PDO. The ability of the spectral bands in distinguishing vinegars by their categories and aging time is mainly based on a series of bands observed in the region of 1500–900 cm^{-1}. These bands provided valuable information about changes related to specific compounds during aging in wood barrels (acetic acid, alcohols, ethers, esters, etc.). Since aging is a

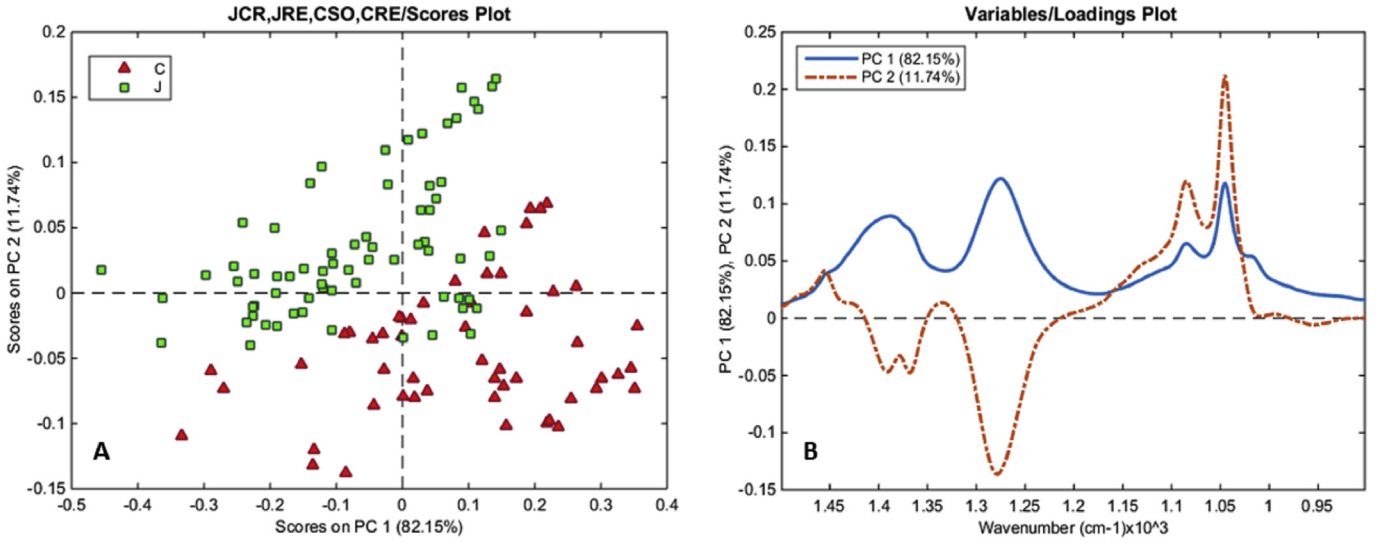

Fig. 5. Results of principal component analysis carried out with ATR-FTIR spectral data of "Vinagre de Jerez" ("J") and "Vinagre Condado de Huelva" ("C") PDOs. The scores plot (A) is shown as well as the corresponding loadings of the first two principal components (PC1 and PC2) (B). The acronyms for the vinegar categories are defined in Table 1.

continuous variable, the intermediate categories in aging (e.g. "Reserva") were overlapped with the other categories to some extent, while the spectral data allowed a clear separation of the most and least aged categories. This procedure also allowed characterizing sweet vinegars ("Pedro Ximenez" category) whose spectra were clearly different compared to aged categories, mostly due to bands assigned to sugars and Maillard compounds (1175–1000 cm^{-1}). Furthermore, the unique characteristics of the Spanish PDO wine vinegars, which directly affect the ATR-FTIR spectra, also allowed the differentiation between PDOs.

The advantages of this method (e.g. fast, non-destructive and with no-sample preparation) would allow implementing this measurement as an additional control for PDO councils and producers to assess the category of the different wine vinegars established in each PDO, and to monitor the aging process with a simple and rapid procedure. The chemical assignment of the bands described in this work and the previous knowledge of vinegar composition provided an additional chemical support to FTIR spectroscopy for this application.

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ARTÍCULO 2

NIR spectroscopy and chemometrics for the typification of Spanish wine vinegars with a protected designation of origin

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ABSTRACT

High-quality wine vinegars protected by the indication "Protected Designation of Origin" (PDO) need efficient tools to protect their brands and prevent adulteration and unfair competition. In this sense, Near-Infrared spectroscopy (NIRs) combined with chemometrics has demonstrated its usefulness in food authentication. This work assessed NIRs and Chemometrics as a rapid and non-destructive methodology for this purpose. In this study, 83 high-quality wine vinegars of the Spanish PDOs "*Vinagre de Jerez*", "*Vinagre de Condado de Huelva*" and "*Vinagre de Montilla-Moriles*" of different categories, and 11 wine vinegars without PDO, were analyzed in the range 12000-4000 cm^{-1}. Principal component analysis (PCA) was performed to explore the spectra and Partial Least Squares-Discriminant Analysis (PLS-DA) was used to build classification models. The high ability of prediction obtained (>90% correct classification) demonstrated the usefulness of this methodology for authentication of PDO wine vinegars and their categories.

1. Introduction

Wine vinegar has become a highly appreciated food product in gastronomy and one of the most consumed types of vinegar in Europe (Paneque, Morales, Burgos, Ponce, & Callejón, 2017). Some wine vinegars, traditionally linked to a specific geographical area, have their specifications related to their chemical and sensory features controlled by European regulations under a legislative system named "Protected Designation of Origin" (PDO) (Chinnici et al., 2009). Thus, as occurring with other food, such as extra virgin olive oil, wine vinegars with a PDO are recognized as a food product with the highest quality. In this field, Spain is one of the major producers of high-quality wine vinegars, producing three of the five types of PDO vinegars in Europe (Council Regulation (EC) No 510/2006): *"Vinagre de Jerez"*, *"Vinagre de Condado de Huelva"* and *"Vinagre de Montilla-Moriles"*. These vinegars are made under traditional processed and from high quality wines protected by their corresponding PDO. Furthermore, some of these PDO wine vinegars are subjected to a period of aging in wooden butts causing chemical modifications in their composition (Morales, Tesfaye, García-Parrilla, Casas, & Troncoso, 2002). According to the sweetness, time and system of aging (*"criaderas and solera"* or *"añada"* systems), different categories are considered within each Spanish PDO (Table 1) having singular and specific characteristics (Council Regulation (EC) No 510/2006).

Due to the demand of high-quality vinegars has significantly increased over the last years, and in addition to food quality is directly related to commercial value, there are suspicious that adulteration and unfair competition in the vinegar industry is being practiced (Consonni, Cagliani, Rinaldini, & Incerti, 2008; Sáiz-Abajo, González-Sáiz, & Pizarro, 2004; Tesfaye, Morales, García-Parrilla, & Troncoso, 2002b). For this reason, wineries and regulatory councils are demanding effective analytical tools to allow rapid and inexpensive analysis to verify the origin of the vinegars in order to protect their brands and to prevent from adulteration. Some of the

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Table 1
PDO and "non-PDO commercial" wine vinegars included in the study.

Wine vinegars	PDO	Category	Aging time	Code	n	Wineries
with PDO	"Vinagre de Jerez" (J)	*Crianza*	>6 months	JCR	15 + 4	34
		Reserva	>2years	JRE	15 + 2	
		Gran Reserva	>10 years	JGR	2	
		Pedro Ximenez	–	JPX	3	
		Total	41			
	"Vinagre de Condado de Huelva" (C)	Sin *crianza*	0 months	CSC	8	8
		Solera	>6months	CSO	9	
		Reserva	>2years	CRE	8	
		Añada	>3years	CAN	4	
		Total	29			
	"Vinagre de Montilla-Moriles" (M)	*Crianza*	>6 months	MCR	4	8
		Reserva	>2years	MRE	4	
		Pedro Ximenez	–	MPX	5	
		Total	13			

Wine vinegars	Origin characteristics	Category	Code	n	Markets
without PDO	Nothern Spain (*Catalonia, La Rioja, Galicia*)		VN	7	4
	Similar geographical area than "*Vinagre de Montilla-Moriles*" PDO	*Crianza, Reserva* and *Pedro Ximenez* categories	VMPX	1	
	No aged	Unknown origin	V	3	3
	Total	11			

classical analytical methods suggested for assessing food quality and differentiating geographical origins such as gas-chromatography-mass spectrometry (GC-MS) (Callejón, Morales, Silva Ferreira, & Troncoso, 2008), atomic absorption spectrometry or high-performance liquid chromatography (HPLC) (Tesfaye, Morales, García-Parrilla, & Troncoso, 2002a), are based on the measurement of the chemical compounds presented in vinegar (e.g. volatiles and phenolic compounds or metals). Although these methods provide high quality information, they require sample pretreatment steps, and they are destructive, time-consuming and expensive. For this reason, there is a growing interest in developing rapid, accurate, inexpensive and non-destructive methodologies based on non-targeted techniques for characterization and authentication of high-quality vinegars (Callejón et al., 2012; De la Haba, Arias, Ramírez, López, & Sánchez, 2014; Fan et al., 2011). In this sense, vibrational spectroscopic techniques, such as Near Infrared spectroscopy (NIRs) and Fourier Transform mid infrared spectroscopy (FTIR) have demonstrated to meet these characteristics being informative at molecular level and very useful for identification and verification of raw materials and final products, producing a single spectral fingerprint of each matrix, and moreover, enable the direct measurement of wine vinegar samples with minimum or no sample preparation (Lohumi, Lee, Lee, & Cho, 2015). Thus, a previous study of the characterization of Spanish PDO wine vinegars by FTIR spectroscopy equipped with an attenuated total reflectance accessory (ATR) (Ríos-Reina, Callejón, Oliver-Pozo, Amigo, & García-González, 2017b) demonstrated the usefulness of this technique in the control of the different categories described in wine vinegar PDOs. This method was applied at first due to it provides a greater amount of chemical information compared to NIR spectroscopy in terms of chemical assignment of observances and allows the interpretation of the spectra without the need of complex chemometrics. Nevertheless, although a direct identification of the compounds is difficult by NIRs, this methodology is extremely useful for highlighting groups of compounds that have more relevance, giving a fingerprint of each sample, as well as it is faster, easier to implement and easy to use (Baeten & Dardenne, 2002; Karoui & De Baerdemaeker, 2007; Stuart, 2004). For all these reasons, NIRs could be a good alternative to be applied and its performance in high-quality wine vinegars authentication must be checked.

However, the fact that the differences between the NIR spectra of different compounds are usually very subtle, and the spectral occurrences in the NIR region are commonly dominated by overtones, combination absorption bands and normally possess broad overlapping, makes necessary the use of chemometrics. Thus, the information provided by NIRs requires advanced multivariate data analysis (such as principal component analysis 'PCA', classification methods as partial least squares-discriminant analysis 'PLS-DA', soft independent modeling by class analogy 'SIMCA', etc.) to allow an efficient treating and interpreting of the signals, as well as to perform a discrimination, classification and authentication of samples. In this context, in the last few years, the use of NIRs in combination with multivariate chemometric analysis has been widely reviewed for many different approaches such as authentication, detecting adulteration or differentiating geographical origins of food products (Alamprese, Amigo, Casiraghi, & Engelsen, 2016; Cozzolino, 2014; Grassi, Amigo, Lyndgaard, Foschino, & Casiraghi, 2014; Liu et al., 2008; Pillonel et al., 2003). However, with regard to the classification of vinegars by NIRs, there are only a few papers related to wine vinegars and high-quality wine vinegars, pointing out the utility of this spectroscopic technique in relation to other food commodities (Casale, Sáiz Abajo, GonzálezSáiz, Pizarro, & Forina, 2006; Fan et al., 2011; Zhao et al., 2011). Furthermore, despite the advantages of NIRs and FTIR vibrational spectroscopic techniques are well known nowadays, the implementation of one of these techniques in the characterization and classification of these high-quality wine vinegars still requires to be further studied, comparing their suitability in the analysis of this food matrix. For these reasons, the aim of the study is to investigate the potential and suitability of NIRs in conjunction with multivariate classification tools as a rapid, inexpensive and non-destructive methodology for the characterization and authentication of the three Spanish wine vinegar PDOs ("*Vinagre de Jerez*", "*Vinagre de Condado de Huelva*" and "*Vinagre de Montilla-Moriles*"), assessing its ability to classify PDO wine vinegars according to their category and to discriminate them from commercial wine vinegars without PDO.

2. Materials and methods

2.1. Samples

2.1.1. PDO wine vinegars

Eighty-three wine vinegar samples belonging to the three Spanish PDOs were analyzed in this study: 41 samples from

"*Vinagre de Jerez*", 29 from "*Vinagre de Condado de Huelva*", and 13 from "*Vinagre de Montilla-Moriles*". These samples were provided by the Regulatory Councils of each Spanish PDO, which assessed and certified the authenticity of the wine vinegars. The less number of samples collected for the PDO "*Vinagre de Montilla-Moriles*", as well as the lack of "*Gran Reserva*" samples, is explained by the fact that it has been recently registered with the indication of PDO (registered in 2015). Furthermore, a different number of samples within each PDO were collected for the established categories (aged and sweet) due to the rate of production of each category during last years (2014-2015). More information about samples included in the study is shown in Table 1.

2.1.2. Commercial wine vinegar samples without PDO

A total amount of 11 wine vinegars from different regions were purchased in local markets and wineries and named in the study as "Commercial samples without PDO" (V): 7 samples produced in northern Spain; 1 wine vinegar from the same region as "*Vinagre de Montilla-Moriles*" PDO but without the PDO indication; and 3 samples without specification of the geographical origin. The number of samples was inevitably limited by their production and availability. Therefore, the work was developed under a feasibility point of view and the production and occurrence in the market was take into account for the construction of the models.

2.2. NIR measurements

NIR spectra were collected in absorption mode using an ABB Bomen IR spectrometer (Q-interline, X, Denmark), equipped with a 1 mm path length cuvette. Spectral data were collected in the range of 12000–4000 cm^{-1}, with a resolution of 8 cm^{-1} and 64 scans for both backgrounds and samples. Wine vinegar samples were directly analyzed without sample pre-treatment by pipetting them into 1 mL shell vial, 40 × 80 mm transparent (Skandinaviska Genetec AB, Lund, Sweden) before measurement. The spectrometer was interfaced to a computer with GRAMS/AI™ Spectroscopy Software (Thermo Fisher Scientific software) for spectral acquisition and exportation. The spectrum of each sample was obtained in triplicate in a random sequence at room temperature (21–23 °C).

2.3. Data processing and multivariate analysis

Data analysis was performed by using PLS_Toolbox 7.9.5 (Eigenvector Research Inc. Wenatchee, WA) working under MATLAB v.8.5.0 environment (The Mathworks Inc. Natick, MA). Different preprocessing methods were studied prior to multivariate data analysis. The best pre-processing method was smoothing (SMT) 7 point and second order filtering operation, to reduce random noise and standard normal variate (SNV) method (Barnes, Dhanoa, & Lister, 1989) to correct for baseline variations due to the different scattering of the samples. Moreover, mean centering (MC) was performed on the spectra. Two segments of the spectrum were removed from the whole wavenumber range of the spectra: the first one because of the low value of the signal/noise and the second one because of the strong combination band of O-H from water (4000-5430 cm^{-1} and 7200-6400 cm^{-1}, respectively). The corrected NIR spectra before and after preprocessing are shown on Fig. 1 (Supplementary Material).

Before classification models, an exploratory analysis of the data is advisable to be performed to detect outliers, recognize patterns in samples distribution and relationships between variables and classes. For this purpose, PCA was carried out prior to any classification approach. After the PCA models, full cross validation (leave-one-out) was used as validation method for the PLS-DA models. Several PLS-DA models were built with different classification purposes: the first one, classifying the different commercialized categories (aged and sweet) within the same PDO, and the second purpose was to differentiate wine vinegars with PDO from those without PDO certification. The models were tested using a data set that was not used in the process of calibration model building. The samples belonging to each dataset were randomly selected by the Kennard-Stone algorithm (Kennard & Stone, 1969) and split into training and test sets (Table 1 Supplementary Material), making sure that in both datasets at least one sample of each category or class was included (with the corresponding replicates). The first dataset (training set) was composed by 75% of the samples (in triplicate) to perform a calibration and internal validation of the models. The other dataset (test set) was composed by 25% of total samples to evaluate the discriminative power of the models. Test samples were only used in the final stage to evaluate the true predictive ability of the calibrated model. The ability of classification of PLS-DA was assessed by statistical parameters as sensitivity, specificity and classification error of calibration (CAL), cross-validation (CV) and prediction (PRED). The correct number of latent variables (LV) was chosen taking into account the root mean square error of calibration (RMSEC) and leave-one-out cross-validation (RMSECV), selecting the number of LVs that led to a minimum of both parameters.

3. Results and discussion

NIR spectra of the wine vinegar samples are shown (Fig. 1A Supplementary Material) as well as the pre-processed spectra (Fig. 1B Supplementary Material). In both cases, the differences between categories or PDOs are not easily observed and further data processing is required. Therefore, PCA was used to explore the spectra. This procedure also allows detecting any grouping of samples. The number of principal components (PCs) was selected according to their explained variance.

3.1. Exploratory analysis of spanish PDO wine vinegars

3.1.1. "Vinagre de jerez" PDO

Two principal components (PCs) were extracted in the PCA model of "*Vinagre de Jerez*" PDO. Fig. 1 shows the score and loading plots obtained of the first two PCs (PC1 and PC2) that explained an accumulative explained variance of 81.21%.

On one hand, Fig. 1A shows that PC1 and PC2 allowed the discrimination between the aging categories, placing samples of less aged vinegars ("*Crianza*" >6 months aged) in the positive side of PC1 and PC2, and placing samples of the most aged category ("*Reserva*" and "*Gran Reserva*") in the negative side of PC1 and PC2. However, some overlapping was also observed between samples belonging to "*Crianza*" (from 6 months to 2 years aged) and "*Reserva*" (more than 2 years aged) categories due to the proximity between their ranges of aging and the fact that time is a continuous variable. Thus, "*Reserva*" vinegars with more years of aging are more similar to vinegars from "*Gran Reserva*" category, and vinegars with only two years of aging are closer to "*Crianza*" vinegars that have to be aged for at least 6 month. In the same way, there was a sample belonging to "*Reserva*" category placed near "*Gran Reserva*" samples (category aged for at least 10 years) that seemed to have an aging longer than the rest of "*Reserva*" wine vinegar samples. There was also a clearly differentiation of the sweet category ("*Pedro Ximenez*") from the rest of the samples. A previous analysis of these PDO wine vinegars by middle infrared and multivariate fluorescence spectroscopy showed similar results (Ríos-Reina et al., 2017a; Ríos-Reina et al., 2017b).

On the other hand, 6 wine vinegars belonging to the "*Vinagre de Jerez*" PDO (4 "*Crianza*" and 2 "*Reserva*") were purchased from the

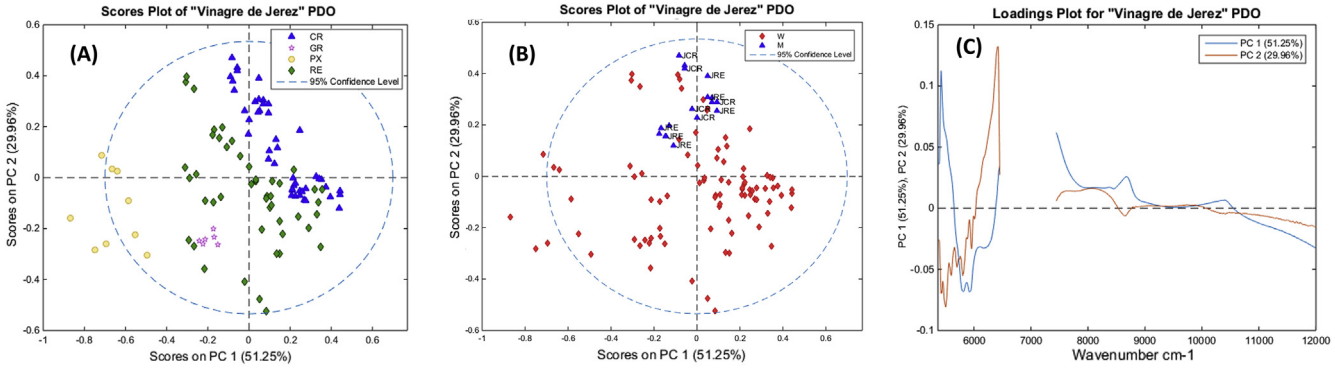

Fig. 1. Results of principal component analysis carried out with NIR spectral data of "*Vinagre de Jerez*" wine vinegars. The scores plots with the representation of the categories **(A)** and with the representation of the provenance of samples **(B)** are shown as well as the corresponding loadings of the first two principal components (PC1 and PC2) **(C)**. The acronyms for the different vinegar categories are defined in Table 1.

market (named "M") instead of collected from the wineries by the regulatory councils (named "W"), and they were included in the PCA model to assess whether they would comply with the requirements and characteristics controlled under the PDO according to their spectral features. The second figure (Fig. 1B) shows that these samples were placed with the rest of the PDO wine vinegars collected from wineries. The observed similarity between these two groups might indicate that their characteristics and quality were in agreement with the PDO regulations. However, it could be observed that one "*Reserva*" sample (showed in the figure in triplicate) was grouped with samples belonging to the immediately before aged category "*Crianza*" instead of with their labeled category, which could indicate a mislabeled sample that aged for a shorter time than the period regulated for a "*Reserva*" category. This result may indicate that NIR could be subjected to another study with the specific objective of labelling verification.

In regards to the loadings (Fig. 1C), the main absorption bands involved in chemical variation during aging, and also related to sweet category, were those from 5200 to 6500 cm^{-1}. These bands have been previously assigned to the presence of water, which shows an intense absorption at ~6860 cm^{-1} (~1450 nm) related to the first O-H overtone of both water and ethanol (Osborne, Fearn, & Hindle, 1993), and in the region of 5300-5000 cm^{-1} (~1900-1950 nm), related to the combination of stretching and deformation of the O-H group in water. The absorption bands around 6000 cm^{-1} (~1690 nm) might be related to the -CH_3 stretching first overtone or C-H groups of chemical compounds that suffer changes during aging, widely described in literature (Callejón, Torija, Mas, Morales, & Troncoso, 2010; García-Parrilla, Heredia, & Troncoso, 1999; Tesfaye, Morales, Benítez, García-Parrilla, & Troncoso, 2004). These absorption bands could be associated to aromatic compounds (Cozzolino, Smyth, & Gishen, 2003; Yu, Fu, Xie, Ying, & Zhou, 2007) which have been found to increase their concentration during aging together with the increasing of phenolic compounds released by wood barrels and oxidation products derived from chemical reactions (Callejón et al., 2008; Morales et al., 2002). Moreover, the absorption band at ~5600 cm-1 (~1790 nm), which is also relevant and related to O-H bonds, has been associated with sucrose, fructose and glucose in fruit juices (Giangiacomo & Dull, 1986). Those compounds are present in high concentration in "*Pedro Ximenez*" vinegars due to the fact that they are produced by the addition of "*Pedro Ximenez*" Sherry wine, which has a high concentration of grape sugars. All these particular characteristics reflected by the spectra provide the chance to discriminate the different commercialized "*Vinagre de Jerez*" PDO categories.

3.1.2. "*Vinagre de condado de huelva*" PDO

Fig. 2 shows the results obtained by carrying out a PCA with wine vinegars belonging to "*Vinagre de Condado de Huelva*" PDO. Firstly, a PCA was carried out with the total of the "*Vinagre de Condado de Huelva*" samples (Fig. 2A). The scores plot of the first two PCs (explaining 92.95% of total variance) allowed a separation of samples according to the aging time with a trend of placing from less aged vinegars (SC and SO) in the negative side of PC1 to the most aged vinegars (RE and AN) in the positive side of PC1 and PC2. It could be observed that 12 wine vinegars collected from two specific wineries (marked with circles) were placed in the most positive side of PC1 and they seemed not to follow the general trend of the PCA model according to the aging time. The analysis was repeated to verify that these samples were not spectral outliers. Moreover, when a PCA model was developed only with these 12 wine vinegars, the aging effect was also observed in the same way (Fig. 2B), placing the most aged samples in the positive side of PC1. The difference of these wine vinegars will probably be a special characteristic of their raw material (i.e. produced by a different wine or from different grape varieties, but always from the allowed ones in this PDO). To study these samples in depth, more accurate analytical techniques (e.g. Nuclear Magnetic Resonance, mass spectrometry, etc.) will be applied.

Once these samples were studied separately, they were removed from the global model and a PCA was again performed to better explore the total dataset (Fig. 2C). The scores plot of the first two PCs allowed the separation of samples according to the aging time (explaining 94.87% of total variance). The intense wavelength region of PC2 had an important role in the discrimination of wine vinegars without aging (SC) and the immediately aged category *"Solera"* (SO) aged for at least 6 months and PC1 showed a clear differentiation between the less aged categories (SC and SO) with less than 2 years of aging, and the most aged categories *"Reserva"* and *"Añada"* (RE and AN) with more than 2 and 3 years of aging, respectively. Results also showed that aging system was also an important difference: "*Añada*" category is obtained by a static aging whereas "*Reserva*" wine vinegars are obtained by the traditional method named "*criaderas y solera*" where vinegars are aged in different butts and they are sequentially mixed. Finally, the scores plot showed two "*Reserva*" wine vinegars placed next to the "*Solera*" samples. This behavior could be explained as these samples were probably aged during the minimum time allowed for the "*Reserva*" category (2 years) and therefore, their characteristics were similar to the most aged "*Solera*" wine vinegars (between 6 months and 2 years of aging).

Regarding the loading plot of PC1 and PC2 (Fig. 2D) for the last

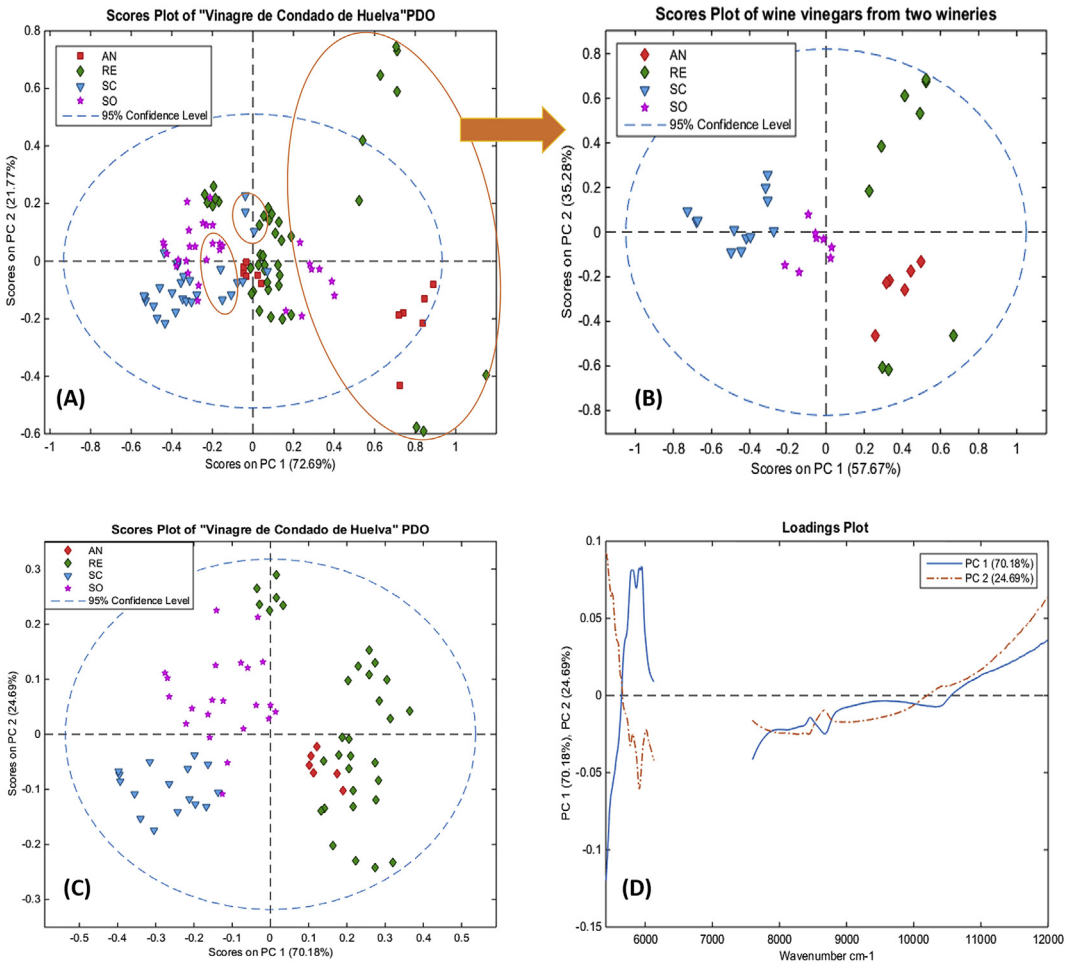

Fig. 2. Results of principal component analysis carried out with NIR spectral data of "*Vinagre de Condado de Huelva*" wine vinegars. The scores plot of the first principal components obtained with the total of wine vinegars is shown **(A)**, as well as the results obtained with only the samples from two specific wineries **(B)** and the scores and loadings plots **(C and D)** after removing the samples from these two specific wineries. The acronyms for the different vinegar categories are defined in Table 1.

PCA model, the region of the spectra that showed the highest relevance in this model (5200-6200 cm^{-1}) agreed with those observed in the developed "*Vinagre de Jerez*" PCA model. Once again, the absorption band at ~5200 cm^{-1} of PC1, previously assigned to the absorption bands of water, explained the distinction of the least aged vinegars whereas the bands located between 5800 and 6200 cm^{-1} explained the separation of the most aged samples. This observation was partially explained by the evaporation process that occurs during aging. Thus, less aged wine vinegars contain more water in the composition since water evaporates during the aging process (Callejón et al., 2010; Tesfaye et al., 2002a,b). This phenomenon gives rise to an increase in the concentration of the rest of compounds (e.g. phenolic and aromatic compounds) whose absorption bands are mainly associated to the bands located between 5800 and 6200 cm^{-1}.

3.1.3. "Vinagre de Montilla-Moriles" PDO

A PCA model was carried out to explore the data and to detect grouping and outliers in "*Vinagre de Montilla-Moriles*" PDO samples (Fig. 3). Despite the low number of samples, the first two PCs also seemed to allow the differentiation of the categories according to aging like the models obtained with the other two PDOs (Fig. 3A). Moreover, "*Pedro Ximenez*" wine vinegar category was again clearly differentiated from the rest of samples by the spectral wavelengths of PC1 (96.83% of total variance). The loading plot showed again the importance of the bands associated to water (at 5200 cm^{-1}) as well as the region where sugars absorbs (5600 cm^{-1}), possibly related to the special composition of "*Pedro Ximenez*" vinegars. Thus, "*Vinagre de Montilla-Moriles Pedro Ximenez*" is produced by adding must of raisins (dried grapes) of the *"Pedro Ximenez"* variety during the production process (Council Regulation (EC) No 510/2006), increasing the concentration of sugars in the vinegar and other compounds such as brown pigments produced by Maillard reaction of the carbohydrates and free amino acids (Casale et al., 2006). In the case of "*Vinagre de Montilla-Moriles*" PDO, the reducing sugar content must be at least 70 g/L (Council Regulation (EC) No 510/2006). However, as the production of "*Pedro Ximenez*" category in the other commercialized PDO "*Vinagre de Jerez*" is different, the final composition may be also quite different. Thus, in the case of "*Vinagre de Jerez*" PDO, sweet vinegars are produced by the addition of "*Pedro Ximenez*" Sherry wine, which entails a content of at least 60 g/L of reducing material from this wine (Council Regulation (EC) No 510/2006). This difference was also observed by developing a PCA model with "*Pedro Ximenez*" samples from both PDOs (Fig. 3C-D), in which samples from "Vinagre de Jerez" and "Vinagre de Montilla-Moriles" were placed separately.

3.1.4. Wine vinegar samples without PDO

In order to corroborate the ability of the developed methodology in the authentication of PDO wine vinegars with respect to

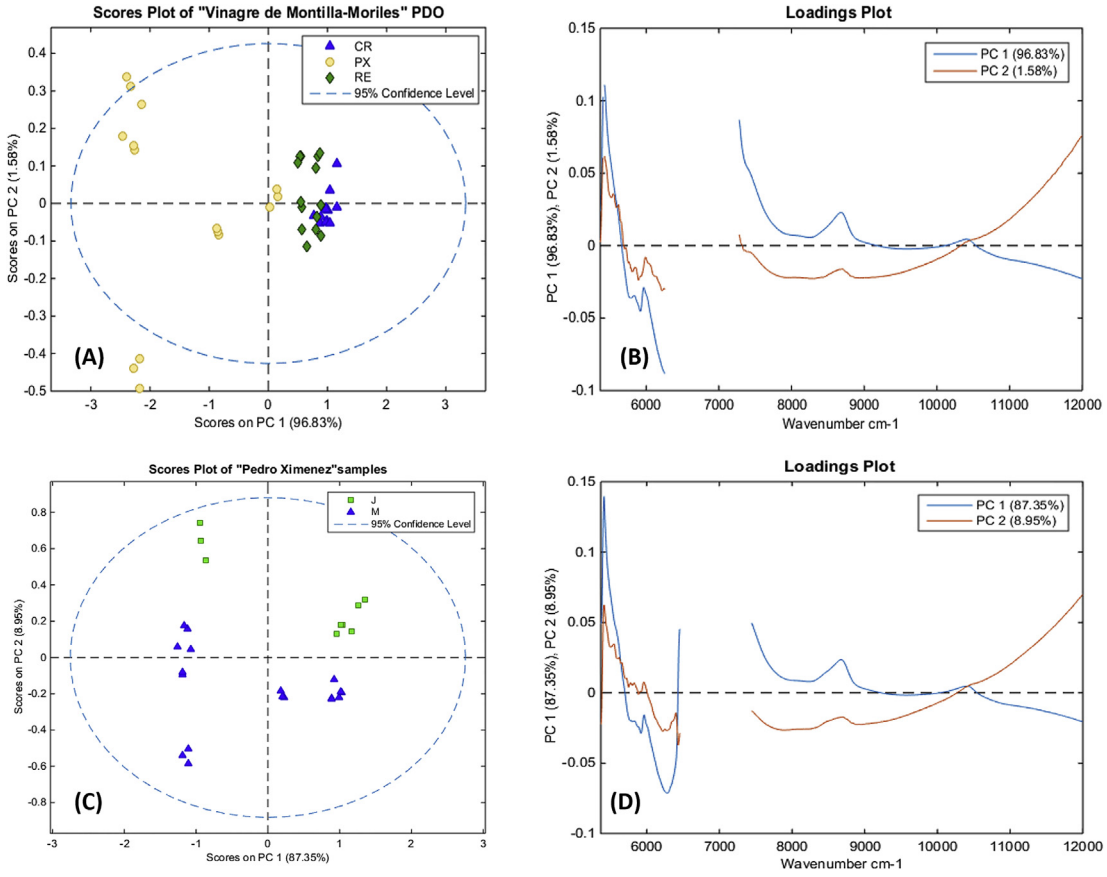

Fig. 3. Results of principal component analysis carried out with NIR spectral data of "*Vinagre de Montilla-Moriles*" samples. The **s**cores and loadings plots of the first two principal components (PC1 and PC2) are shown **(A and B),** as well as the results of principal component analysis (scores and loadings plots) of the first two principal components (PC1 and PC2) carried out with "*Pedro Ximenez*" wine vinegar samples **(C and D)**. The acronyms for the different wine vinegar categories and PDOs are defined in Table 1.

non-PDO wine vinegars, some samples without a PDO indication (V) were included in the models together with the wine vinegars of each PDO ("*Vinagre de Jerez*" "J", "*Vinagre de Condado de Huelva*" "C" and "*Vinagre de Montilla-Moriles*" "M"). As a first step, different PCA models were built (Fig. 2 Supplementary Material). The score plots showed a clear difference between the PDO wine vinegars and the group of vinegars without PDO. Only the visual differentiation between some "*Pedro Ximenez*" samples belonging to "*Vinagre de Montilla-Moriles*" PDO (MPX) and one "*Pedro Ximenez*" wine vinegar without PDO (VPX) was not perfectly clear (Fig. 2 C.1 Supplementary Material). However, a "*Pedro Ximenez*" sample without PDO that was produced in the same geographical area as "*Vinagre de Montilla-Moriles*" PDO (VMPX) was placed in the scores plot extremely separated from "*Pedro Ximenez*" vinegars within the PDO (MPX). These results reaffirms the unique quality and characteristics of wine vinegars produced under the specifications of a PDO due to major controls and their traditional method of production that provided high-quality conditions to vinegars since a very long period of time is required. No use of NIRs technology on the differentiation of PDO wine vinegars from vinegars without the PDO indication has been reported to date.

Regarding loadings plot (Fig. 2 A.2, B.2, C.2 Supplementary Material), the first two PCs, which explained between 94% and 99% of total variance in the three PCA models, pointed out that the spectral regions mainly responsible of the differentiation were again those between 5000 and 6500 cm^{-1} together with the region between ~10000 cm^{-1} and ~12000 cm^{-1} that had an important relevance in this particular case.

3.2. PLS-DA classification models

After a preliminary exploratory analysis of spectra by PCA, PLS-DA model was developed for a classification purpose. The first PLS-DA models were developed to classify samples between their established PDO categories (henceforth "*category classification*"). The second PLS-DA models were developed to confirm the ability of NIRs to authenticate and differentiate PDO wine vinegars from wine vinegars without the PDO designation (henceforth "*PDO/origin classification*"). Categories with lower number of samples were not included in the models. Moreover, "*Añada*" category was grouped with "*Reserva*" category in "*Vinagre de Condado de Huelva*" PDO, as the aging time regulated in each one was similar (more than two-three years of aging), differing only on the aging system used. The models were tested by dividing the total number of samples in two sets (training and test sets). Further information about the number of samples used for modeling and predicting are shown in Table 1 Supplementary Material.

3.2.1. PLS-DA models for distinguishing the three aged categories within each PDO (category classification)

The statistical parameters obtained by PLS-DA in the different models are shown in Table 2a. High sensitivity and specificity values (%) were obtained in the PLS-DA models for each category. The 87-100% of the samples were correctly classified, demonstrating that all the categories within each PDO could be successfully separated from the rest of classes. These results confirmed and improved those obtained in a previous study of the authors (Ríos-

Table 2
Sensitivity, specificity and classification errors (%) obtained for (a) PLS-DA classification models corresponding to the vinegar category of each Spanish PDO; (b) PLS-DA classification models to differentiate PDO wine vinegars from external vinegars. The acronyms for the different vinegar categories are defined in Table 1.

(a) "Category classification"

Spanish PDOs	"Vinagre de Jerez"			"Vinagre de Condado de Huelva"			"Vinagre de Montilla-Moriles"		
Nº LVs	6			2			5		
Category	JCR	JRE	JPX	CSC	CSO	CRE-CAN	MCR	MRE	MPX
Sensitivity CAL	94.4	88.6	100.0	100.0	93.3	100.0	100.0	100.0	100.0
Sensitivity CV	94.4	85.7	100.0	100.0	93.3	100.0	88.9	88.9	100.0
Sensitivity PRED	100.0	88.9	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Specificity CAL	97.6	88.1	98.6	97.4	82.4	100.0	100.0	095.2	100.0
Specificity CV	95.1	83.3	95.8	100.0	93.3	100.0	100.0	85.7	100.0
Specificity PRED	83.3	100.0	100.0	100.0	73.3	91.7	100.0	100.0	100.0
Class. Error CAL	3.9	11.6	0.7	1.3	12.1	0.0	0.0	2.3	0.0
Class. Error CV	5.2	15.5	2.1	1.3	12.1	0.0	5.5	12.6	0.0
Class. Error PRED	8.3	5.5	0.0	0.0	13.3	4.1	0.0	0.0	0.0

(b)"PDO/origin classification"

	"Vinagre de Jerez"PDO	Wine vinegars without PDO	"Vinagre de Condado de Huelva" PDO	Wine vinegars without PDO	"Vinagre de Montilla-Moriles" PDO	Wine vinegars without PDO
Nº LVs	3		4		3	
Sensitivity CAL	100.0	100.0	100.0	100.0	100.0	100.0
Sensitivity CV	100.0	100.0	100.0	100.0	100.0	95.8
Sensitivity PRED	100.0	100.0	100.0	100.0	100.0	100.0
Specificity CAL	100.0	100.0	100.0	100.0	100.0	100.0
Specificity CV	100.0	100.0	100.0	100.0	95.8	100.0
Specificity PRED	100.0	100.0	100.0	100.0	100.0	100.0
Class. Error CAL	0.0	0.0	0.0	0.0	0.0	0.0
Class. Error CV	0.0	0.0	0.0	0.0	2.1	2.1
Class. Error PRED	0.0	0.0	0.0	0.0	0.0	0.0

Reina et al., 2017a), in which these vinegars were analyzed by multidimensional fluorescence spectroscopy coupled with different classification tools, resulting in the need of using a non-linear classification tool (support vector machines) to obtain good classification results. In the case of NIRs, a linear classification approach is enough for obtaining good results. However, the results also showed that the categories that showed lowest classification rates were those that would be in the boundaries between categories according to their aging period ("*Solera*" for "*Vinagre de Condado de Huelva*" PDO and "*Reserva*" in the other two PDOs). These results were acceptable considering the high variability of these samples due to the factor that they were aged over a wide range of time that is reflected over their complex chemical composition (García-Parrilla et al., 1999). These intermediate categories were expected to be spectroscopically and chemically similar to the vinegars of the immediately previous or following category. Furthermore, the highly variability in the intermediate categories was also observed by Callejón et al. (2012), whose study revealed that the lowest classification rates were obtained for the intermediate aged category "*Reserva*", with an aging time between "Crianza" and "Gran Reserva" categories.

3.2.2. PLS-DA classification of PDO wine vinegars and wine vinegars without PDO (PDO/origin classification)

After the exploratory PCA analysis, a PLS-DA was applied to confirm the ability of NIRs to authenticate and differentiate PDO wine vinegars from those without the PDO indication. PLS-DA results are shown in Table 2b. The low classification errors of prediction obtained in the models demonstrated that a good separation of PDO wine vinegar samples from those without the PDO certification could be performed with the proposed methodology. As the number of samples between the two groups (with and without PDO) was not properly balanced for building robust models, several PLS-DA models were developed and tested with the same number of samples per group. Samples from the PDO group (11 different samples each time) were randomly selected and included in the models together with samples without PDO. The results obtained matched with those shown in Table 2b. These results highlighted the unique characteristics conferred by the high quality of raw wines used (each belonging to the corresponding PDO), the traditional system of production and aging of the Spanish PDO wine vinegars ("*criaderas and solera*" or "*añada*" systems), and the standardize procedure of production. All of these characteristics, together with the routine controls by the regulatory councils, allowed a rapid classification and differentiation from the rest of wine vinegars without a PDO indication. Although other researches showed the utility of NIRs in the differentiation of vinegars with different raw materials (Sáiz-Abajo et al., 2004), or even between different wine vinegar manufacturing methods (De la Haba et al., 2014), no references have been found showing the differentiation and classification of Spanish PDO wine vinegars from vinegars without a PDO by using only NIRs.

3.2.3. Comparison of classification results obtained between NIRs and FTIR analysis

In order to explore the potential and advantages of using NIRs and ATR-FTIR spectroscopy and their suitability for PDO wine vinegar classification, the performance of the two techniques was compared for a wine vinegar classification purpose (classification of categories within a PDO). For this purpose, PLS-DA results obtained by both spectroscopic techniques were examined by comparing the percentage of correct predictions (Table 3). For this comparison,

Table 3
Comparison of PLS-DA models obtained for the classification of wine vinegar samples according to the commercialized category within each PDO. The percentage of correct predictions correspond to models tested with independent data sets.

PLS-DA Classification of categories within each PDO (category classification)			
Spectroscopic technique		NIR	FTIR
Spectral region used		12000-7200 cm^{-1} and 6400-5430 cm^{-1}	1500-900 cm^{-1}
Nº variables included		1450	310
Pre-processing technique		SMT + SNV	–
PLS-DA model of "Vinagre de Jerez" PDO	LVs	6	4
	Correct prediction of categories (%)	JCR: 91.7	JCR: 83.75
		JRE: 94.5	JRE: 70.6
		JPX: 100.0	JPX: 100.0
PLS-DA model of "Vinagre de Condado de Huelva" PDO	LVs	2	2
	Correct prediction of categories (%)	CSC: 100.0	CSC: 70.0
		CSO: 86.7	CSO: 81.7
		CRE-AN: 95.9	CRE-AN: 80.0
PLS-DA model of "Vinagre de Montilla-Moriles" PDO	LVs	5	5
	Correct prediction of categories (%)	MCR: 100.0	MCR: 91.7
		MRE: 100.0	MRE: 58.4
		MPX: 100.0	MPX: 100.0

PLS-DA classification models were built with ATR-FTIR data obtained in a previous research carried out by the authors of this study (Ríos-Reina et al., 2017b). In this model, the range between 1500 and 900 cm^{-1} was included in the PLS-DA, due to the fact that, as it was previously reported, it showed the main spectral bands assigned to complex interacting vibrations related to the unique fingerprint of each vinegar.

NIR classification models showed percentages of correct predictions in the range 86.7–100% in most of the categories while in the case of ATR-FTIR the percentage of correct prediction were 58.4–100% (Table 3). In most of the cases, the classification rates were higher in NIRs compared to ATR-FTIR. However, it is important to consider the advantages of both techniques. Thus, ATR-FTIR spectroscopy has the advantages of being able to determine absorption bands with clear chemical assignments, which facilitates the interpretation of the spectra. Although NIR spectra are more difficult to interpret and the calibration procedures were more complicated, it also shows an easy and robust analysis and yielded satisfactory classification results for PDO wine vinegars. Depending on the classification purpose (categories and PDO vs non-PDO) and the needs for interpreting the spectra, one of both techniques could be proposed to be applied in quality control of vinegars, or even the combination of both of them.

4. Conclusions

In this work, the combination of NIR with chemometrics has demonstrated to be useful for a rapid characterization and classification of the Spanish PDO wine vinegars and for controlling the authenticity of their commercialized categories (aged and sweet). A simple exploration of the NIR data by a PCA pointed out some that aging and the protection under a PDO had an effect in the spectra, showing similarities between the spectra of the aged categories of the three Spanish PDOs. The absorption bands most involved in aging changes, and also related to sweet category, were those from ~5200 to ~6500 cm^{-1}, associated to the presence of water and aromatic and phenolic compounds that have shown changes during aging. Furthermore, the sweet category "*Pedro Ximenez*" showed some characteristic bands at the same region (~5600 cm-1) mainly associated to sugars, due to their special characteristic of production. The unique characteristics of the Spanish PDO wine vinegars, which directly affect to the NIR spectra, allowed a satisfactory classification according to the category (aged and sweet categories within each PDO) and PDOs versus non-PDO differentiation (PDO wine vinegars from vinegars without this quality indication) by the development of PLS-DA classification models with the NIR spectrum of samples.

The advantages of this methodology would allow implementing it as an alternative tool for fingerprinting wine vinegar samples on a large scale, this analytical tool being cost-effective and rapid. Further research will be carried out to test this technique at industrial scale with a higher number of samples to evaluate the efficiency in real authentication problems.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.foodcont.2018.01.031.

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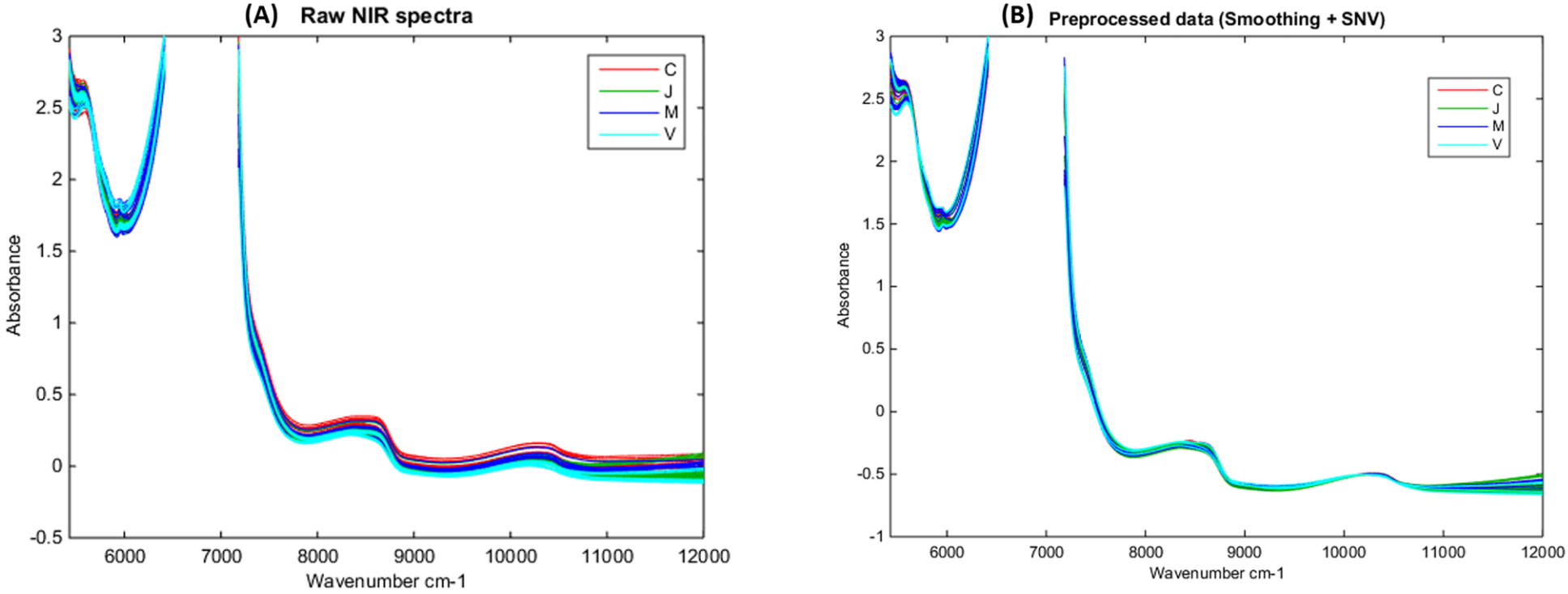

Figure I. NIRs spectra (with section spectra removed) of all PDO wine vinegars included in the study before (raw spectra) **(A)** and after preprocessing (smoothing and SNV) **(B)**. Note: C= "*Vinagre de Condado de Huelva*", J= "*Vinagre de Jerez",* "M"= "*Vinagre de Montilla-Moriles*", V= "Wine vinegars without PDO"

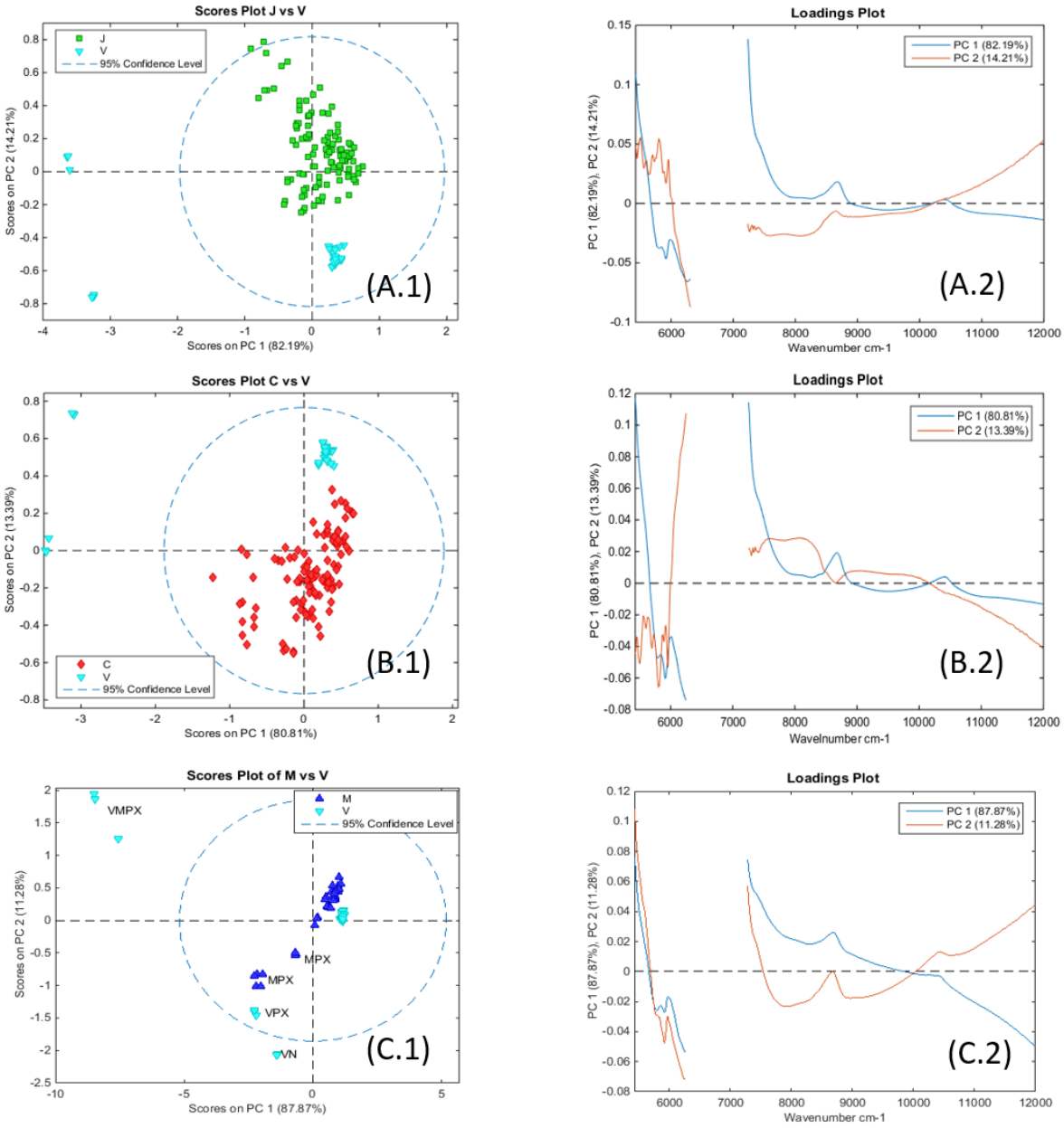

Figure II. Scores plots obtained by principal component analysis carried out with NIR spectra of "*Vinagre de Jerez*" **(A.1)**, "*Vinagre de Condado de Huelva*" **(B.1)**, "*Vinagre de Montilla-Moriles*" **(C.1)**, together with wine vinegars without PDO. The loadings plots of the first two principal components (PC1 and PC2) **(A.2, B.2, C.2)** are also shown. Note: C= "*Vinagre de Condado de Huelva*", J= "*Vinagre de Jerez",* "M"= "*Vinagre de Montilla-Moriles*", V= "Wine vinegars without PDO"

Table I. Number of samples (by triplicate) used in each dataset for developing PLS-DA classification models. The acronyms for the different vinegar categories are defined in Table 1.

Model	**Classification of wine vinegar categories within each Spanish PDO**											
Spanish PDO	***"Vinagre de Jerez"***				***"Vinagre de Condado de Huelva"***				***"Vinagre de Montilla-Moriles"***			
Category	**JCR**	**JRE**	**JPX**	**Total**	**CSC**	**CSO**	**CRE-CAN**	**Total**	**MCR**	**MRE**	**MPX**	**Total**
Model	36	36	6	78	12	15	18	45	9	9	12	30
Prediction	9	9	3	21	6	6	6	18*	3	3	3	9
Model	**Classification of Spanish PDO wine vinegars from wine vinegars without PDO**											
Category	***"Vinagre de Jerez" PDO vs* wine vinegars without PDO**				***"Vinagre de Condado de Huelva" PDO vs* wine vinegars without PDO**				***"Vinagre de Montilla-Moriles" PDO vs* wine vinegars without PDO**			
Spanish PDO	**J**		**V**	**Total**	**C**		**V**	**Total**	**M**		**V**	**Total**
Model**	90/24		21/24	111/48	63/24		21/24	72/48	30/24		24/24	54/48
Prediction**	30/9		12/9	42/18	24/9		12/9	36/18	9/9		9/9	18/18

*Note: Samples belonging to the two specific wineries (2SC, 2SO, 2 Re and 2 AN) marked in Figure 3.B were not included in the PLS-DA models. **All the samples included in training and test sets/ balanced number of samples per group included in training and test sets.

Acronyms J= "*Vinagre de Jerez*"; C= "*Vinagre de Condado de Huelva*"; M= "*Vinagre de Montilla-Moriles*"; V= *wine vinegars without PDO*.

BLOQUE I:

CARACTERIZACIÓN Y CLASIFICACIÓN ESPECTROSCÓPICA DE LOS VINAGRES DE VINO ESPAÑOLES CON DOP

CAPÍTULO II:
ESPECTROSCOPÍA DE FLUORESCENCIA MULTIDIMENSIONAL

CHAPTER II.

Multidimensional fluorescence spectroscopy

RESUMEN

En este capítulo, las muestras de vinagres de vino con DOP fueron analizadas por espectroscopia de fluorescencia multidimensional (EFM), también conocida como espectroscopia de fluorescencia de excitación-emisión, en combinación con técnicas quimiométricas, con dos objetivos: caracterizar y autentificar estos vinagres con DOP y sus correspondientes categorías tal y como se muestra en el primer trabajo de este capítulo, y por otro lado, detectar y cuantificar la cantidad de caramelo de mosto adicionado a estos vinagres, cuyos resultados se presentan en el segundo trabajo. Esta técnica fue seleccionada debido a que también es un método analítico rápido, económico y efectivo, que no requiere manipulación de la muestra y que está creciendo como técnica competitiva para el análisis de alimentos, ya que proporciona en pocos segundos una matriz de datos de excitación-emisión que puede ser usada como huella dactilar del producto.

En el primer estudio, publicado en Food Chemistry 230 (2017) 108–116, se analizaron 79 muestras de vinagre de vino de las tres DOP españolas de varios productores y abarcando las diferentes categorías: 30 “Vinagre de Jerez”, 18 “Vinagre de Montilla-Moriles” y 21 “Vinagre de Condado de Huelva”. Las matrices de excitación-emisión obtenidas estaban formadas por los rangos de λex de 250 a 680 nm (cada 5 nm) y de λem de 310 a 800 nm (cada 2 nm). Sobre estas matrices se construyeron modelos PARAFAC para extraer la información relevante y para poder a partir de ella, construir modelos de clasificación robustos y fiables mediante análisis de discriminación de mínimos cuadrados parciales (PLS-DA) y Máquinas de vectores de soporte (SVM) para: (1) diferenciar las categorías de vinagre de vino pertenecientes a la misma DOP, y (2) diferenciar categorías similares de vinagre de vino que pertenecen a diferentes DOPs.

Una evaluación visual de los perfiles EEM de fluorescencia señaló ciertas tendencias en las diferentes categorías de vinagre, comunes para las tres DOP: a mayor envejecimiento de los vinagres, los máximos espectrales se desplazaban hacia longitudes de onda mayores. Por otra parte, la categoría dulce “Pedro Ximénez" mostró máximos de excitación y emisión incluso en longitudes de onda mayores que las categorías de envejecimiento, probablemente debido al mayor contenido en azúcares de estos vinagres. Los fluoróforos potenciales extraídos por el análisis PARAFAC y su contribución en cada DOP y categorías de vinagre de vino estudiadas, permitieron construir mediante PLS-DA y SVM, modelos de clasificación capaces de discriminar las diferentes categorías de vinagre de vino dentro de cada DOP, obteniéndose mejores resultados con el modelo SVM (> 92% de clasificación). Además, los modelos SVM también

fueron capaces de diferenciar las DOP españolas para categorías de vinagre similares, debido a diferencias espectrales relacionadas con las distintas materias primas y el origen de cada DOP.

Por tanto, los resultados obtenidos demostraron que el uso de la metodología propuesta y las herramientas quimiométricas (espectroscopía de excitación por fluorescencia acoplada al modelado PARAFAC y el método de clasificación SVM) son una perfecta combinación para extraer la información química más relevante de los vinagres de vino, así como para clasificar y discriminarlos considerando sus correspondientes categorías o DOP registradas.

Además, como se muestra en el segundo trabajo de este capítulo, publicado en Food Chemistry 287, 115–125, la espectroscopía de fluorescencia multidimensional combinada con técnicas quimiométricas también fue estudiada para la detección y cuantificación del caramelo de mosto adicionado a estos vinagres de calidad. Esta adición es una práctica común en la producción del vinagre de vino con el fin de corregir y unificar el color final de diferentes lotes. Aunque la legislación actual lo permite, es interesante disponer de un método rápido que permita la cuantificación del caramelo de mosto, ya que la adición del mismo al vinagre de vino puede suponer un fraude cuando se utiliza para simular el efecto de un envejecimiento más prolongado y vender ese producto como tal. En este contexto, el objetivo de este segundo trabajo fue evaluar la fluorescencia multidimensional como una técnica rápida y rentable para detectar y cuantificar el caramelo de mosto en vinagre.

Para ello se analizaron por esta técnica, bajo las mismas condiciones del trabajo anterior, un set de muestras que incluía: vinagres de vino de dos de las DOPs españolas “Vinagre de Jerez” y “Vinagre de Montilla-Moriles” (16 de la categoría Crianza y 18 de la categoría Reserva), pertenecientes al trabajo anterior, así como 4 muestras de vinagre de vino de estas dos DOPs sin caramelo adicionado recolectadas de la propia bodega. Además, se adicionaron diferentes cantidades de caramelo de mosto (MO-7) diluido a los distintos tipos de muestras de vinagre anteriormente mencionados. Tras el análisis, se analizaron y compararon diferentes técnicas multivariantes de análisis de datos, como el Análisis de Factor Paralelo (PARAFAC), análisis discriminante y regresión de mínimos cuadrados parciales (NPLS-DA, PLS-DA y NPLS), con el fin de seleccionar la mejor metodología para detectar/diferenciar la presencia/ausencia de caramelo de mosto en el vinagre de vino, así como cuantificarlo. Para controlar y demonstrar la validez de los resultados, las muestras se analizaron por HPLC, tomando como referencia algunos compuestos descritos como característicos del caramelo de mosto, como es el 5-hydroxymethylfurfural (5-HMF).

Los resultados demostraron que la fluorescencia multidimensional combinada con un método quimiométrico adecuado puede ser una herramienta valiosa para detectar y, por primera vez, cuantificar la adición de caramelo de mosto a vinagre de vino sin tratamiento de muestra (errores de predicción bajos, RMSEP≈0.24). Además, los resultados mostraron que no solo el 5-HMF es característico de este caramelo de mosto como se pensaba en un principio, sino que hay otros compuestos aún no identificados que aumentan a medida que se aumenta la cantidad adicionada de caramelo de mosto.

Por otro lado, este estudio demostró que, ambas metodologías de clasificación y regresión estudiadas (de múltiples vías y a partir de PARAFAC) proporcionaron buenos resultados. Sin embargo, la metodología de múltiples vías (NPLS y NPLS-DA) tiene la ventaja de ser más sencilla y rápida, pero por otro lado no proporciona la misma cantidad de información sobre los compuestos fluorescentes como proporciona PARAFAC.

Además, se realizaron pruebas sensoriales triangulares, cuyos resultados demostraron que, con incluso a bajas concentraciones, la adición de caramelo de mosto al vinagre de vino tenía un efecto sobre las características organolépticas, reafirmando la necesidad de disponer de un método que permita realizar una determinación rápida, barata y eficaz de la adición de caramelo de mosto en estas muestras.

Finalmente, cabe destacar que este trabajo abre un nuevo campo a estudiar, en el que se necesita profundizar más en el tema, con el objetivo de establecer un límite o crear un protocolo de control con respecto a la adición del caramelo de mosto a los vinagres con DOP.

ARTÍCULO 3

Characterization and authentication of Spanish PDO wine vinegars using multidimensional fluorescence and chemometrics

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ABSTRACT

This work assesses the potential of multidimensional fluorescence spectroscopy combined with chemometrics for characterization and authentication of Spanish Protected Designation of Origin (PDO) wine vinegars. Seventy-nine vinegars of different categories (aged and sweet) belonging to the Spanish PDOs "*Vinagre de Jerez*", "*Vinagre de Montilla-Moriles*" and "*Vinagre de Condado de Huelva*", were analyzed by excitation-emission fluorescence spectroscopy. A visual assessment of fluorescence landscapes pointed out different trends with vinegar categories. PARAllel FACtor analysis (PARAFAC) extracted the potential fluorophores and their values in the PDO vinegars. This information, coupled with different classification methods (Partial Least Square Discrimination Analysis "PLS-DA" and Support Vectors Machines "SVM"), was able to discriminate the wine vinegar category within each PDO, for which SVM models obtained better results (>92% of classification). In each category, SVM also allows the differentiation between PDOs. The proposed methodology could be used as an analysis method for the authentication of Spanish PDO wine vinegars.

1. Introduction

Vinegar is a product used worldwide as a condiment and food preserving agent, obtained by a double fermentation process (alcoholic and acetic fermentation) of sugary and starchy substrates (FAO, 1998). Vinegar can be produced by different methods and raw materials (such as malt, apple, rice, etc.), among which wine vinegar is the most commonly produced and consumed vinegar in Mediterranean countries and Central Europe (Polo & Sanchez-Luengo, 1991).

For many years, wine vinegar has been considered as a low-cost secondary product spontaneously derived from wine production. However, in recent years wine vinegar has become a valued food product much appreciated in gastronomy. As a result, the demand for high-quality wine vinegars has significantly increased over the last years. In this framework, Spain is one of the major producers of high-quality wine vinegars, including three of the five types of vinegar registered in Europe (Council Regulation (EC) No 510/2006) with a Protected Designation of Origin (PDO): "*Vinagre de Jerez*", "*Vinagre de Montilla-Moriles*" and "*Vinagre de Condado de Huelva*" (Table I Supplemental Material). The production of these high-quality PDO wine vinegars in Spain is centered in Andalusia, each of them made from the corresponding protected wines (*Jerez, Montilla-Moriles and Condado de Huelva*), which provides singular and specific characteristics to each vinegar. In addition, the production of high-quality vinegars requires an aging period in wooden butts. During the period of aging, some chemical modifications take place providing them with unique organoleptic properties and higher sensory quality (Morales, Tesfaye, García-Parrilla, Casas, & Troncoso, 2002). According to the sweetness, time and method of aging ("*criaderas and solera*" and "*añada*" system), different categories are considered within each Spanish PDO (Table 1).

The longer aging time is directly related to both the higher quality and production costs of these wine vinegars. This fact increases the final market price and makes the quality assurance and authentication of the Spanish PDO wine vinegars an important issue. For this reason, objective analytical methodologies are required to guarantee the wine vinegar authenticity and fight

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Table 1
Wine vinegar samples analyzed according to the Spanish PDOs

Protected designation of origin (PDO)	Vinegar category	Category name	Acronym	Aging time (months)	Number of samples*
"*Vinagre de Jerez*"	Aged	"Vinagre de Jerez"	JCR	≥6	13
		"Reserva"	JRE	≥24	11
		"Gran Reserva"	JGR	≥120	2
	Sweet	"Pedro Ximenez"	JPX	–	4
"*Vinagre de Montilla-Moriles*"	Aged	"Crianza"	MCR	≥6	7
		"Reserva"	MRE	≥24	4
		"Gran Reserva"	MGR	≥120	2
	Sweet	"Pedro Ximenez"	MPX	–	5
"*Vinagre de Condado de Huelva*"	Non-aged	"Vinagre Condado de Huelva"	CSC	0	6
	Aged	"Viejo Solera"	CSO	≥6	6
		"Viejo Reserva"	CRE	≥24	7
		"Viejo Añada"	CAN	≥36	2

* *Note:* Each sample corresponds to different producers.

against frauds. However, the most common analytical techniques used for the characterization and authentication of these vinegars rely on chromatographic methods that are often expensive and time-consuming (Aceña, Vera, Guasch, Busto, & Mestres, 2011; Cirlini, Caligiani, Palla, & Palla, 2011). Thus, in recent years there has been a growing interest in developing rapid, inexpensive, non-destructive and direct methodologies based on non-targeted techniques for food authentication. Fluorescence spectroscopy has been increasingly applied as a competitive, high sensitivity, fast and non-destructive technique in food analysis (Karoui & Blecker, 2011). This spectroscopic technique has been more commonly used in wine (Airado-Rodríguez, Galeano-Díaz, Durán-Merás, & Wold, 2009; Azcarate et al., 2015), but rarely adopted for wine vinegar samples (Callejón et al., 2012) and hence, there is still scarce information about vinegar fluorescent components.

In this sense, wine vinegar is a very complex multi-component mixture of chemical compounds due to its traditional making procedure, the raw material used and the aging period and method employed. Some of these chemical compounds are polyphenols, amino acids and vitamins (Airado-Rodríguez, Durán-Merás, Galeano-Díaz, & Wold, 2011), whose presence is related to the wine chemical basis. To handle this complexity, fluorescence multidimensional measurements, such as excitation-emission fluorescence spectroscopy, combined with adequate multi-way methods (Andersen & Bro, 2003; Sádecká & Tóthová, 2007) have been proven to be useful for characterization of complex food matrices (Callejón et al., 2012; Christensen, Becker, & Frederiksen, 2005; Elcoroaristizabal et al., 2016; Lenhardt, Bro, Zeković, Dramićanin, & Dramićanin, 2015). Measuring the emission spectra at different excitation wavelengths results into a bi-dimensional Excitation-Emission Matrix (EEM), which contains unique information of each measured sample. Therefore, a three dimensional array is obtained when all the samples are gathered together, so requiring an appropriate data processing for its interpretation.

An adequate multiway method, such as PARAllel FACtor Analysis (PARAFAC), can be used to decompose fluorescence EEMs into different independent groups of fluorescence components (fluorophores), as well as their relative concentration (scores) in each sample (Bro, 1997). The information provided by the resolved fluorophores has been successfully applied in food quality control, since it can reveal clearer insights into the relationships between the intrinsic food properties and the quality of the product. For instance, EEM-PARAFAC has been applied for monitoring the changes occurring during the storage and production of different food samples (Christensen et al., 2005; Elcoroaristizabal et al., 2016) and their characterization (Lenhardt et al., 2015; Tena, Aparicio, & García-González, 2012). Furthermore, the information obtained after EEM data decomposition by PARAFAC modelling could be coupled with different classification methods in order to characterize and classify different food products or detect fraudulent samples (Callejón et al., 2012).

There are numerous classification algorithms such as Partial Least Square Discrimination Analysis (PLS-DA), K-Nearest Neighbors (KNN), Support Vector Machines (SVM) and Soft Independent Modelling of Class Analogy (SIMCA) (Cover & Hart, 1967; Vapnik, 1999; Wold, 1966; Wold, 1976). Among them, Partial Least Squares-Discriminant Analysis (PLS-DA) and Support Vectors Machines (SVM) are two of the most common used ones. PLS-DA is a supervised class-modelling method used for building linear discriminant models (Nocairi, Qannari, Vigneau, & Bertrand, 2005), which has been successfully applied to a wide variety of food matrices for classification purposes (Azcarate, Cantarelli, Pellerano, Marchevsky, & Camiña, 2013; Lenhardt et al., 2015; Liu, He, & Wang, 2008). The main advantage of the PLS-DA approach is the ability of handling highly collinear and noisy data. However, one of the main issues is that PLS-DA models need a sufficient and balanced amount of samples for each class; and sometimes it is difficult to acquire sufficient samples of some classes, due to their cost of production or their non-availability in the market. Moreover, classes that are not effectively separated linearly are common in food products. Support Vector Machines (SVM) is an effective non-linear machine learning technique suitable for both classification and regression analysis (Xu, Zomer, & Brereton, 2006). In comparison to PLS-DA, the main advantage of SVM is its flexibility in modelling complex classification problems that are non-linear. A common disadvantage of SVM is the lack of transparency of the results, since there are no statistics such as scores and loadings available for easy visualization.

Several researchers have tested the SVM's performance in different food authentication problems obtaining better results than other traditional classification methods. For instance, Acevedo, Jiménez, Maldonado, Domínguez, and Narváez (2007) observed that SVM performed better than SIMCA, k-NN, and PLS-DA for discrimination of wines according to their PDO, which also enabled the selection of the most relevant UV–Vis wavelengths for samples classification. In the same way, Callejón et al. (2012) proved that SVM in conjunction with excitation-emission fluorescence spectroscopy was a more adequate methodology than PLS-DA for the classification of sherry vinegars according to their aging time. However, the aforementioned study was only focused on the classification of a limited number of wine vinegar categories (aged vinegars) belonging to one Spanish PDO ("*Vinagre de Jerez*").

In this context, the aim of this work was to investigate the feasibility of using excitation-emission fluorescence spectroscopy combined with several chemometric techniques for characterization and classification of the three Spanish PDOs wine vinegars

and their commercialized categories. First, EEM data will be analyzed by PARAFAC in order to characterize spectroscopically and chemically different commercialized wine vinegar categories (aged and sweet) according to each Spanish PDO. Then, these results will be used to build reliable classification models able to differentiate between the wine vinegar categories corresponding to each Spanish PDO, and each PDO within the same wine vinegar category.

2. Materials and methods

2.1. Wine vinegar samples

Seventy-nine wine vinegar samples from the three Spanish PDOs coming from several producers were analyzed in this study (Table 1): 30 "*Vinagre de Jerez*", 18 "*Vinagre de Montilla-Moriles*" and 21 "*Vinagre de Condado de Huelva*" samples. Among the aged categories, these vinegars are aged by the traditional system called "*criaderas and solera*", except for the "*Vinagre de Condado de Huelva Añada*" which is aged by using the static aging system called "*añada*". Regarding the "*Pedro Ximenez*" category, it should be highlighted that this sweet category differs from the aged category not only by the aging time but also by other factors such as their different production process. Thus, they are produced by the addition of must of raisined "Pedro Ximenez" grapes (in the case of "Vinagre de Montilla-Moriles") or the addition of "Pedro Ximenez" wine to the vinegar. All the samples were purchased from local wineries working in compliance with current regulations of each Spanish PDO. The samples were collected in triplicate and stored in amber vials at room temperature until the analysis.

Within each PDO, a different number of samples were collected for the established categories (aged and sweet) according to the production/sale rates of each category during the last years (2014–2015). In these years, the general trend for the three Spanish PDOs reveals a higher production of the categories with less aging time due to market trends. For instance, "*Vinagre de Jerez*" (JCR) represented approximately 60% of total sales in the PDO "*Vinagre de Jerez*", while sales of "*Vinagre de Jerez Reserva*" (JRE) and "*Vinagre de Jerez Gran Reserva*" (JGR) accounted for 40% and 1% of the total, respectively. Similarly, "*Vinagre de Condado de Huelva*" (CSC) category had the highest sales growth up 38% of the total. Meanwhile among the aged categories of "*Vinagre de Condado de Huelva*" PDO, the most commercialized vinegar categories were, in decreasing order: "*Solera*" (CSO), "*Reserva*" (CRE) and "*Añada*" (CAN). In the same way, "*Vinagre de Montilla-Moriles Crianza*" (MCR) was the most commercialized one of the "*Vinagre de Montilla-Moriles*" PDO due to the recent incorporation to the Spanish PDOs.

2.2. Fluorescence analysis

Fluorescence measurements were recorded using a Varian Cary-Eclipse fluorescence spectrophotometer (Varian Iberica, Madrid, Spain), equipped with two Czerny-Turner monochromators, and a Xenon discharge lamp pulsed at 80 Hz with a half peak height of ~2 μs (peak power equivalent to 75 kW). A high-performance R298 photomultiplier tube detector was used for collection of the fluorescence spectra. Wine vinegar samples were directly analyzed without sample pre-treatment by pipetting them into 3.5 mL quartz cuvettes before measurement. Standard quartz cells (Hellma Analytics, Müllheim, Germany) of 1 cm path length were used to carry out the measurements in a Peltier thermostatted cuvette holder (25.00 ± 0.05 °C). The spectrometer was interfaced to a computer with Cary-Eclipse software for spectral acquisition and exportation.

The fluorescence Excitation-Emission Matrices (EEMs) were obtained by varying the excitation wavelength (λ_{ex}) ranging between 250 and 700 nm (every 5 nm), and recording the emission spectra (λ_{em}) from 300 to 800 (every 2 nm). For these measurements, excitation and emission slits were both set at 5 nm, and the scan rate was fixed to 1200 nm min^{-1}. The system was wavelength calibrated every day by means of the water Raman peak to account for a possible wavelength drift of the instrument. EEMs were registered by triplicate for each sample and preprocessed in order to avoid noisy and non-informative areas by selecting shorter spectral ranges (λ_{ex} from 250 to 680 nm, and λ_{em} from 310 to 800 nm).

2.3. Software and data analysis

EEM data analysis was performed by using the PLS_Toolbox 7.9.5 (Eigenvector Research Inc., Wenatchee, WA) working under Matlab v.8.5.0 environment (The Mathworks Inc., Natick, MA). Before the analysis, EEMs data were corrected for –Rayleigh and Raman scattering (Elcoroaristizabal, Bro, García, & Alonso, 2015) – by removing and replacing the scattering areas with interpolated values by using the FLUCUT function included in the PLS_Toolbox. FLUCUT Removes Rayleigh scattering (and possibly Raman) by inserting NaN and 0 values in Excitation-Emission Matrices (EEMs) where the Rayleigh bands are. Alternatively, FLUCUT may also be used to generate weights that can be used for deweighting (instead of eliminating) these regions.

2.3.1. Parallel factor analysis (PARAFAC)

PARAllel FACtor models were performed on the corrected EEM data in order to extract the relevant information and develop models for: (1) different wine vinegar categories belonging to the same Spanish PDO, and (2) similar wine vinegar categories belonging to different Spanish PDOs.

Before modelling, the EEM landscapes corresponding to the same Spanish PDO (1) were rearranged into a three-dimensional structure ($\underline{\mathbf{X}}$) of size (3 replicated samples × λ_{em} × λ_{ex}): 90 × 246 × 87 for the PDO "*Vinagre de Jerez*"; 54 × 246 × 87 for the PDO "*Vinagre de Montilla-Moriles*", and 63 × 246 × 87 for the PDO "*Vinagre de Condado de Huelva*". In a similar way, the EEM landscapes corresponding to similar wine vinegar categories but different Spanish PDOs (2) were organized into a three-way array ($\underline{\mathbf{X}}$) of size (3 replicated samples × λ_{em} × λ_{ex}): 78 × 246 × 87 for "*Crianza*"; 66 × 246 × 87 for "*Reserva*" category, and 27 x 246 × 87 for "*Pedro Ximenez*" category. No PARAFAC analysis was carried out for the "*Gran Reserva*" category due to the limited number of samples.

Then, each three-way dataset ($\underline{\mathbf{X}}$) was decomposed by PARAFAC (Bro, 1998). The proper number of factors for each model was determined by using the CORe CONsistency DIAgnostic test (CORCONDIA) (Bro & Kiers, 2003), the percentage of variance explained by the model and the visual inspection of the recovered spectral profiles and residuals. Non-negative constraints for all modes (concentrations and both spectral profiles) were applied to obtain meaningful chemical solutions.

2.3.2. Classification methods

Partial Least Squares-Discriminant Analysis (PLS-DA) (Nocairi et al., 2005) and Support Vectors Machines (SVM) (Vapnik, 1999) algorithms were used to build classification models for discrimination of the wine vinegar categories within each Spanish PDO. On the one hand, PLS-DA is a classification method based on partial least squares regression (PLS) that transforms the data into a set of linear latent variables for predicting the dependent or class

variable, making models that allow the maximum separation among classes. The class variable forms a so-called dummy matrix that indicates whether a sample belongs to a certain class or category. In our study, three different wine vinegar categories were considered in each Spanish PDO, therefore, the dimensions of each dummy matrix was 3×3.

On the other hand, SVM is a relative new chemometric tool based on the statistical learning theory (SLT). It is a supervised learning method that searchers for the optimal separating hyperplane between the different data classes by maximizing the distance between the hyperplane and the closest samples of the training set (the support vectors), keeping the classification error as low as possible (Xu et al., 2006). Only two parameters need to be tuned in SVM, including C (cost) and the kernel parameter γ in Gaussian kernel function. C is a tuning parameter, which weights in-sample classification errors and controls the generalization ability of an SVM. Moreover, within the different kernel functions, an appropriate γ parameter is related to a stable generalization performance. Furthermore, this method does not need a large number of samples to be trained, it is not affected by the presence of outliers and it has been successfully applied to solve a variety of practical classification problems (Acevedo et al., 2007; Liu et al., 2008; Xu et al., 2006).

As the results obtained in our study for the classification of wine vinegar categories within each PDO showed that SVM developed better classification models, only Support Vectors Machines (SVM) was used to build classification models for distinguishing the Spanish PDOs in each similar wine vinegar category ("*Crianza*", "*Reserva*" and "*Pedro Ximenez*"). For both approaches, scores of each sample from PARAFAC models were used, 95% confidence intervals were considered for the classification models and vinegar samples were randomly divided into two groups.

The first group of samples (training set), comprising the 75% of samples, was used for calibration and internal validation of the models by means of a venetian blinds cross-validation procedure. For discrimination of the wine vinegar category within each Spanish PDO, this dataset (samples analyzed in triplicate) was formed by 63 ("*Vinagre de Jerez*"), 33 ("*Vinagre de Montilla-Moriles*"), and 39 ("*Vinagre de Condado de Huelva*") samples. Meanwhile, this dataset consisted of 54 ("*Crianza*"), 48 ("*Reserva*"), and 18 ("*Pedro Ximenez*") samples, in order to distinguish the Spanish PDO corresponding to each wine vinegar category.

The second group with the remaining samples (test set) was used as external independent dataset to evaluate the discriminative power of the models (external validation). This dataset was formed by 25% of the samples, and consisted of (samples analyzed in triplicate): 21 ("*Vinagre de Jerez*"), 15 ("*Vinagre de Montilla-Moriles*"), and 18 ("*Vinagre de Condado de Huelva*") samples, in order to discriminate the wine vinegar category within each Spanish PDO. For differentiating the Spanish PDO in each wine vinegar category, this dataset was formed by (samples analyzed in triplicate): 24 ("*Crianza*"), 18 ("*Reserva*"), and 9 ("*Pedro Ximenez*") samples. Afterwards, the samples belonging to each dataset were randomly selected making sure that in both datasets at least one sample, with the corresponding replicates, of each category/PDO was included. Samples belonging to "*Gran Reserva*" and "*Añada*" categories in each PDO (JGR, MGR and CAN) with a low number of samples (≤ 2) were not used. Further information about the number of samples used in each case for calibration and external validation can be found in Table II (Supplementary Material).

The statistical assessment of the quality of both classification models was carried out by means of the comparison of the sensitivity, specificity and classification error of calibration (CAL), cross-validation (CV) and prediction (PRED) parameters (Margraf, Santos, de Andrade, van Ruth, & Granato, 2016) according to Eqs. (5) and (6):

$$\text{Sensitivity}(\%) = [\text{TP}/(\text{TP} + \text{FN})] * 100\%$$

$$\text{Specificity}(\%) = [\text{TN}/(\text{TN} + \text{FP})] * 100\%$$

whereby TP and TN represent the number of samples correctly classified as their real class (e.g. the number of JCR samples predicted as JCR and the number of MCR samples predicted as MCR samples, respectively). On the other hand, FP and FN represent the number of samples misclassified (e.g. the JCR samples assigned to MCR class and MCR samples assigned to JCR class, respectively).

3. Results and discussion

3.1. Fluorescence landscapes of the Spanish PDO wine vinegars

Typical fluorescence landscapes of several samples belonging to the different wine vinegar categories of each Spanish PDO are shown in Fig. 1 (after removing and replacing the first and second order Rayleigh scattering). As it can be observed, the shape of the EEM spectra varies within the same Spanish PDO, which allows us to confirm *a priori* differentiation according to the wine vinegar category (aged or sweet).

A visual assessment of the fluorescence features of the aged categories points out a general tendency for the spectral maxima to be shifted towards longer excitation and emission wavelengths with the aging of the vinegars. Furthermore, similar fluorescence maxima were observed for the different Spanish PDOs wine vinegars according to the aging period. In general, vinegars with a minimum of 6 months of aging (JCR, MCR and CSO) show their maximum peaks at 370/450 nm for both excitation/emission wavelengths ($\lambda_{ex}/\lambda_{em}$), whereas the maximum peaks corresponding to the "*Reserva*" category (JRE, MRE and CRE) appear at higher wavelengths, around 370–470 nm of λ_{ex} and 470–550 nm of λ_{em}. Finally, samples belonging to the most aged categories (JGR, MGR and CAN) show their maxima at 470–500 nm of excitation and 550–600 nm of emission wavelengths, following the general observed trends. The spectral features of the wine vinegars without aging period (CSC), which shows maximum peaks at the shortest wavelengths (around 370/440 nm $\lambda_{ex}/\lambda_{em}$), also confirm this tendency. Interestingly, some samples of the "*Reserva*" category (e.g. "*Vinagre de Montilla-Moriles*" PDO, "*Reserva*" sample "*MRE*" in Fig. 1) show two different maximum peaks probably due to the broader aging period, which can vary from 24 to 120 months ("*Vinagre de Jerez*" PDO and "*Vinagre de Montilla-Moriles*" PDO) or even to longer periods ("*Vinagre de Condado de Huelva*" PDO) (Table 1). These observed spectral features are probably related to the different chemical complexity of the aged categories. In fact, a similar fluorescence trend with the aging of wine samples was observed by Airado-Rodríguez et al. (2011), whose fluorescence landscapes showed a tendency to increase the emission at longer wavelengths with the aging of the wine samples, due an increase in concentration of fluorescence substances (Airado-Rodríguez et al., 2011).

In contrast, the fluorescence landscapes of the sweet vinegar categories, named as "*Pedro Ximenez*" (JPX and MPX) show a highly intense fluorescent area between 550–570 nm and 600–650 nm of excitation and emission wavelengths, respectively. Indeed, these sweet vinegars show their excitation and emission maxima even at longer wavelengths than the ones corresponding to the aged categories. This phenomenon could be explained by the different production and composition of the sweet vinegars in comparison with the aged categories. Thus, the sweet vinegars are produced with the addition of "*Pedro Ximenez*" Sherry wine in the case of "*Vinagre de Jerez*" PDO (containing at least 60 g/L of reducing material from this wine) (Council Regulation (EC) No 510/2006), or adding must of raisined "*Pedro Ximenez*" grapes during the maturing process for the "*Vinagre de Montilla-Moriles*" PDO (Council Regulation (EC) No

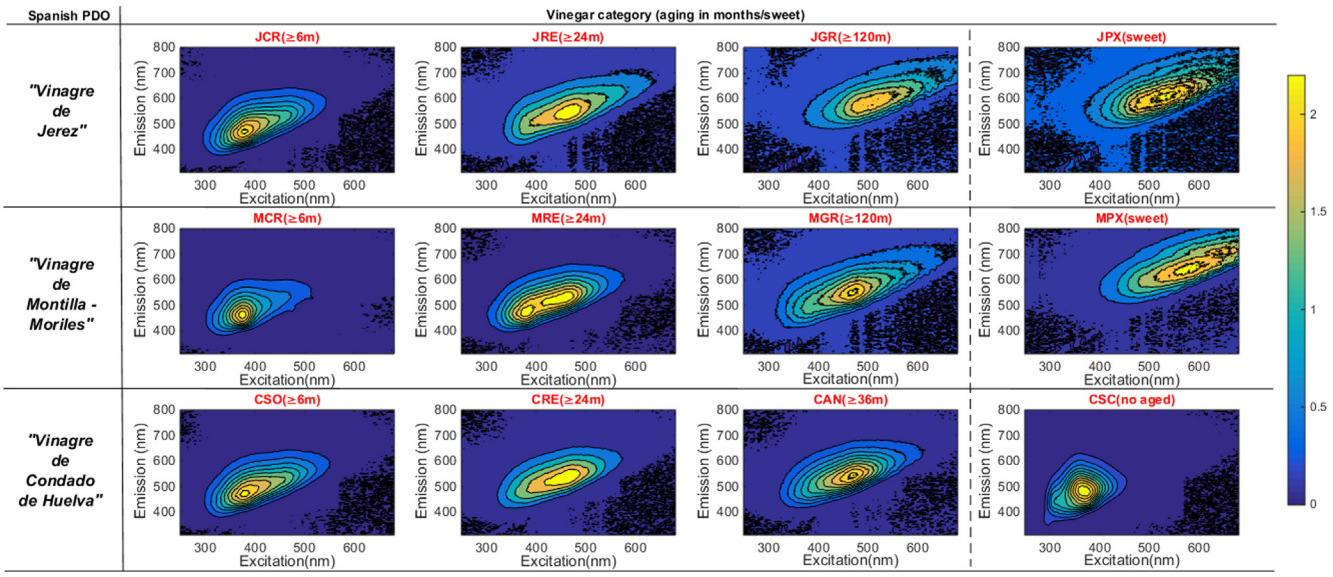

Fig. 1. Excitation-Emission fluorescence landscapes obtained for different categories of Spanish PDO vinegars. The color scale of fluorescence intensity (in arbitrary units) varies from dark blue (lowest signal intensity) to yellow (highest signal intensity).The acronyms for the different wine vinegar categories are defined in Table 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

510/2006). These sweet vinegars have a high carbohydrate content (glucose and fructose) and other compounds (brown pigments and volatile compounds) produced by a Maillard reaction of the carbohydrates and free amino acids (Casale, Sáiz Abajo, González Sáiz, Pizarro, & Forina, 2006), which may be responsible for the observed fluorescence at longer excitation and emission wavelengths.

From these observations, it is clear that the fluorescence landscapes of these Spanish PDO vinegars contain several fluorophores that are highly overlapped in both excitation and emission spectra. In this sense, further decomposition of EEM spectra by PARAFAC will help to clarify the potential fluorophores present in each vinegar category.

3.2. Potential fluorophores of the Spanish PDO wine vinegars

Three individual PARAFAC models were built in order to extract the excitation and emission profiles of the main fluorophores present in the Spanish PDO vinegars (as described in Section 2.3.1). The optimum number of factors for each PARAFAC model was selected comparing the quality parameters of the models built for an increasing number of factors (ranging from one to seven). Specifically, the best PARAFAC models obtained for each Spanish PDO were 5-factor PARAFAC models for the "*Vinagre de Jerez*" and "*Vinagre de Montilla-Moriles*" PDOs, and a 4-factor PARAFAC model for the "*Vinagre de Condado de Huelva*" PDO. The obtained models were enough robust, explaining more than 99% of the variance with a core consistency over zero (Table III Supplementary Material), and represented the underlying chemical spectra of the fluorophores present in these vinegars. Fig. 2 includes the PARAFAC loadings (excitation and emission spectra) of the extracted factors present in each Spanish PDO vinegar, whose corresponding fluorescence emission and excitation maxima are listed in Table IV (Supplementary Material). The fluorescent loading patterns of the modelled factors can be matched to fluorophores described in the literature (Airado-Rodríguez et al., 2011; Dufour, Letort, Laguet, Lebecque, & Serra, 2006; Elcoroaristizabal et al., 2016). However, it is important to note that vinegar contains a wide variety of naturally occurring fluorescent compounds, being each emission/excitation profiles a sum of related fluorescent molecules and not only to a single one (Airado-Rodríguez et al., 2011). The difference in these modelled factors is probably related to the different chemical composition of these vinegars as a consequence of the different raw materials (wines), production and aging processes for each Spanish PDO. This is corroborated by the variation in the score values of the fluorophores modelled for each Spanish PDO according to the vinegar category (Table V Supplementary Material).

The first factor (F1, blue line in Fig. 2) of the PDO "*Vinagre de Jerez*" has a maximum excitation centered at 465 nm and a maximum emission around 535 nm. According to Airado-Rodríguez et al. (2011), this factor could be related to vitamin B2 and its principal forms such as riboflavin, Flavin mononucleotide (FMN), and Flavin adenine dinucleotide (FAD). In contrast, F1 appears at lower wavelengths, specifically at 375/460 nm and 370/470 nm ($\lambda_{ex}/\lambda_{em}$), for the "*Vinagre de Montilla-Moriles*" and "*Vinagre de Condado de Huelva*" PDOs, respectively. In these PDOs, and taking into account these wavelengths, F1 could be due to the presence of coumarins, tannins and other unknown fluorescent compounds originating from wooden casks (Tóthová & Sádecká, 2008), as well as phenols and flavonols, usually in abundance in these vinegars and naturally presented in wines (Airado-Rodríguez et al., 2011; Sádecká & Tóthová, 2007).

The second factor (F2, red line in Fig. 2) has a similar profile for the three PDOs with an excitation and emission maxima between 400–420 nm and 480–505 nm respectively. 5-Hydroxymethylfurfural (HMF), which has been determined in vinegars as a product being formed during the Maillard reaction (García Parrilla, Heredia, & Troncoso, 1999), could match with the wavelengths of F2 according to Zhu, Ji, Eum, and Zude (2009). Furthermore, caramel, which is frequently added to vinegars as a colorant, showed a maximum excitation/emission wavelength at 390–410/482–498 nm according to Sádecká, Tóthová, and Májek (2009) and Tóthová and Sádecká (2008). Its presence in these vinegars could be also related to this second factor.

The third factor (F3, yellow line in Fig. 2) of "*Vinagre de Jerez*" PDO is a peak centered at 500 nm (λ_{ex}) and 580 nm (λ_{em}). This component could be associated to brown pigments produced by some acetic bacteria strains present in vinegar (Polo & Sanchez-Luengo, 1991) since they showed similar excitation/emission wavelengths. However, in "*Vinagre de Montilla-Moriles*" PDO, this factor F3 shows excitation and emission maxima around 470/550 nm, which agrees with the presence of vitamin B2 and

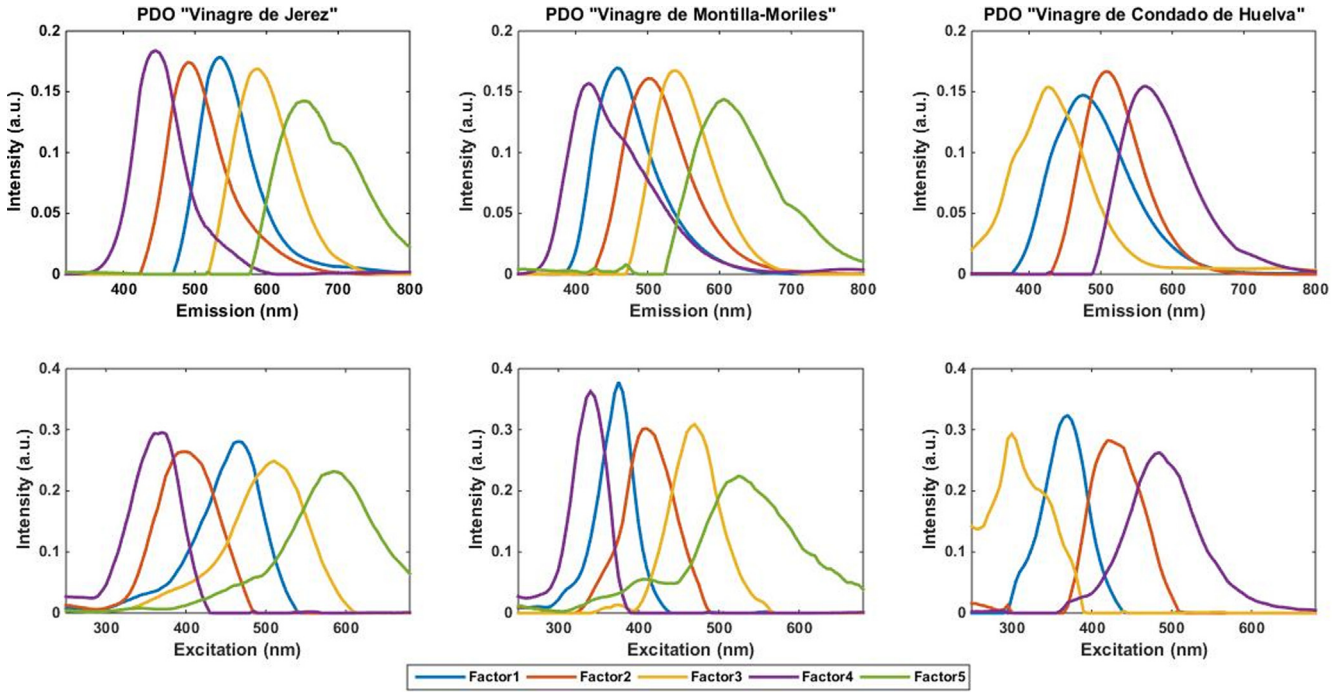

Fig. 2. Excitation and Emission spectra (PARAFAC loadings) of the main fluorophores present in the vinegars of the three Spanish PDOs.

its principal forms (Lenhardt et al., 2015). Finally, the third factor of "*Condado de Huelva*" PDO is centered at 300/425 nm ($\lambda_{ex}/\lambda_{em}$), and these wavelengths could be associated with the phenolic compounds present in these vinegars (Rodríguez-Delgado, Malovaná, Pérez, Borges, & García Montelongo, 2001).

The fourth factor (F4, purple line in Fig. 2) has 340/420 nm and 350/440 nm of excitation and emission maxima for the "*Vinagre de Montilla-Moriles*" and "*Vinagre de Jerez*" PDOs. According to the literature, the excitation/emission wavelengths of this factor could be related to phenolic compounds, the best known fluorescent molecules naturally present in wine that differ in accordance to the grape variety and the vinegar aging. This group of compounds includes phenolic acids and phenolic aldehydes, as well as oxidation and Maillard reaction products (present due to browning processes and oxidative mechanisms taking place during aging and storage), which have shown maximum excitation/emission wavelengths around 330/420 nm (Airado-Rodríguez et al., 2011; Azcarate et al., 2015; Callejón et al., 2012; Dufour et al., 2006; Elcoroaristizabal et al., 2016; Sádecká & Tóthová, 2007). In contrast, in "*Condado de Huelva*" PDO this F4 presents its excitation and emission maxima around 485/560 nm ($\lambda_{ex}/\lambda_{em}$), and it could be associated with the aforementioned vitamin B2.

Finally, a fifth factor (F5, green line in Fig. 2) appears only for the "*Vinagre de Montilla-Moriles*" and "*Vinagre de Jerez*" PDOs, showing excitation and emission maxima at 530/605 nm and 585/655 nm ($\lambda_{ex}/\lambda_{em}$), respectively. There are not reported fluorophores matching exactly with this emission/excitation profile. However, it seems to be related to the special characteristics of the category "*Pedro Ximenez*" for which a higher mean values of this factor was detected in this category (Table V Supplementary Material). The absence of this factor in the "*Vinagre Condado de Huelva*" model also confirms this hypothesis since this sweet category is not registered in this PDO.

3.3. Vinegar category classification within each Spanish PDO

Two different approaches, Partial Least Squares Discriminant Analysis (PLS-DA) and Support Vector Machines (SVM), were used for the development of classification models of Spanish PDO vinegars according to their category. In all cases, the best PLS-DA models were obtained using two latent variables (LVs). This optimum number of LVs was chosen based on the Root Mean Square Error of Cross-Validation (RMSECV), the ROC curves and de variance captured. On the other hand, the optimal parameters for the optimization of SVM models, $\log_{10}(C)$ and $\log_{10}(\gamma)$, were found to be 2 and between −2 and −0.5, respectively. The statistical assessment of the performance of both classification models was carried out by calculating and comparing different classifiers (described in Section 2.3.2) such as sensitivity, specificity and classification error of calibration (CAL), cross-validation (CV) and prediction (PRED). These statistical results are shown in Table 2.

Regarding the PLS-DA models, high sensitivity and specificity values were obtained for the sweet category (JPX and MPX) of "*Vinagre de Jerez*" and "*Vinagre de Montilla-Moriles*" PDOs. The classification errors of prediction obtained for these categories (100% of the samples correctly classified), demonstrated that these samples can be successfully separated from the rest of classes. This is probably due to their different chemical and fluorescence spectral features, since this sweet category emitted at the longest wavelengths (Fig. 1). In a similar way, concerning the non-aged category (CSC) of "*Vinagre de Condado de Huelva*" PDO, the different fluorescence profile observed in the landscapes and the higher mean values of F1 and F3 with respect to the rest of categories (Table V Supplementary Material), could be related to the lowest errors of classification obtained for this category (25.0% of the samples misclassified). However, unsatisfactory results were obtained for the rest of aged categories within each Spanish PDO: JCR, JRE, MCR, CSO and CRE. The low sensitivity and specificity values (mainly under 65.0%) and high classification errors obtained in terms of prediction (between 25.0 and 63.0%), confirm the difficulty in correctly classifying these aged vinegar categories ($\geq$6 and $\geq$24 months) by using linear classification models. This could be related to the similar score values followed by the modelled factors of these categories within each Spanish PDOs (Table V Supplementary Material). Among them, the $\geq$6 months aged samples (JCR, MCR and CSO) were the worst classified ones in all the Spanish

Table 2
Sensitivity, specificity and classification errors (%) obtained for SVM and PLS-DA classification models corresponding to the vinegar category of each Spanish PDO.

Spanish PDO	*"Vinagre de Jerez"*						*"Vinagre de Montilla-Moriles"*						*"Vinagre de Condado de Huelva"*					
Classification model	SVM			*PLS-DA*			SVM			*PLS-DA*			SVM			*PLS-DA*		
Category	JCR	JRE	JPX	JCR	JRE	JPX	MCR	MRE	MPX	MCR	MRE	MPX	CSC	CSO	CRE	CSC	CSO	CRE
Sensitivity CAL	92.9	92.3	100.0	38.7	100.0	100.0	100.0	100.0	100.0	80.0	100.0	100.0	83.3	100.0	100.0	75.0	100.0	100.0
Sensitivity CV	100.0	100.0	100.0	38.7	100.0	100.0	100.0	100.0	100.0	80.0	66.7	100.0	100.0	100.0	100.0	75.0	100.0	100.0
Sensitivity PRED	92.9	92.3	100.0	10.0	66.7	100.0	100.0	100.0	100.0	50.0	100.0	100.0	83.3	100.0	100.0	50.0	100.0	100.0
Specificity CAL	100.0	94.4	96.3	90.9	32.5	94.5	100.0	100.0	100.0	83.3	75.0	100.0	100.0	92.3	100.0	100.0	33.3	62.5
Specificity CV	100.0	100.0	100.0	90.9	25.0	94.5	100.0	100.0	100.0	83.3	62.5	95.8	100.0	100.0	100.0	100.0	25.9	62.5
Specificity PRED	100.0	94.4	96.3	75.0	18.2	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	91.7	100.0	100.0	0.0	25.0
Class. Error CAL	0.0	0.0	0.0	35.1	33.7	2.7	0.0	0.0	0.0	18.3	12.5	0.0	0.0	0.0	0.0	12.5	33.3	18.7
Class. Error CV	0.0	0.0	0.0	35.1	37.5	2.7	0.0	0.0	0.0	18.3	35.4	2.1	0.0	0.0	0.0	12.5	37.0	18.7
Class. Error PRED	3.5	6.6	1.8	62.5	57.5	0.0	0.0	0.0	0.0	25.0	0.0	0.0	8.3	4.1	0.0	25.0	50.0	37.5

PDO vinegar models, showing classification errors until 63.0%. This may be explained due to these wine vinegars are aged over a wide range of time (from 6 to 24 months). Thus, those samples aged until 24 months are expected to be spectroscopically and chemically quite similar to the vinegars of the following category (≥24 months). Similar results were obtained by Callejón et al. (2012).

In contrast, higher sensitivity and specificity levels were obtained for all Spanish PDO vinegars using SVM models (Table 2). The optimal parameters for the optimization of SVM models, $\log_{10}$(C) and $\log_{10}$ (γ), were optimized in the traditional way by using an independent test set (Christiani & Shawe-Taylor, 2000). Between 92% and 100% of the samples were correctly classified in all categories. Even more, all samples belonging to the Spanish PDO "*Vinagre de Montilla-Moriles*" were perfectly classified. Further information about the misclassified category samples within each Spanish PDO is summarized in the confusion matrices shown in Table VI (Supplementary Material). These results also point out that SVM does not need a large number of samples to make a good model, as occurs in our study with some categories, and further, it is not affected by the presence of outliers. These results demonstrated that this methodology could be successfully used for the authentication of the vinegar category belonging to each Spanish PDO.

3.4. Spanish PDO classification within similar vinegar categories ("Crianza", "Reserva" and "Pedro Ximenez")

In this classification task, the objective was to classify the samples by their PDO for a single category. Three PARAFAC models were built according to the vinegar categories under study, i.e. "*Crianza*", "*Reserva*" and "*Pedro Ximenez*", in order to discriminate their corresponding Spanish PDO. In a similar way to the previous sections, the best PARAFAC models obtained for each vinegar category were selected comparing the quality parameters of the models, which are shown in Table VII (Supplementary Material). In this case, a 4-factor PARAFAC model was obtained for "*Crianza*", while a 5-factor PARAFAC model was built for "*Reserva*", and a 3-factor PARAFAC model was constructed for "*Pedro Ximenez*" category. The obtained models explained more than 97.0% of the variance with a core consistency over zero. The related PARAFAC loadings (excitation and emission spectra) obtained for the models corresponding to each vinegar category are illustrated in Fig. 3.

The maxima wavelengths (λ_{ex} and λ_{em}) of the different factors obtained for the "*Crianza*", "*Reserva*" and "*Pedro Ximenez*" PARAFAC models match with the different fluorophores described in detail in Section 3.2. As shown in Fig. 3, the "*Crianza*" category is characterized by factors with excitation and emission ranges between 340–500 nm and 430–580 nm, respectively. These factors are mainly related to fluorescent compounds naturally presented in high concentration in wine such as phenols, flavonols and vitamins as previously described (Section 3.2). These compounds have a higher contribution in this category (Table V Supplementary Material) since the less aged wine vinegars retain more compounds coming from the raw materials. Regarding the "*Reserva*" samples, a higher number of factors were required to model this category, i.e., more fluorescent compounds with a wide range of excitation/emission spectra were needed to describe its underlying chemical composition. In this case, the longer aging period undergone by vinegars of the "*Reserva*" category plays a crucial role in this chemical complexity. There is an enrichment of these vinegars with more phenolic compounds (released by the wood barrels) and oxidation products (derived from the development of certain chemical reactions among vinegar components), whose concentration levels have been proven to increase during the aging process (Callejón, Morales, Silva Ferreira, & Troncoso, 2008). The wavelengths of the factors modelled for "*Pedro Ximenez*" category are associated to fluorophores emitting at the highest excitation and emission wavelengths (upper than 475 and 550 nm, respectively). However, more information, not available in the literature, is needed to identify these fluorophores.

For all the categories under study, the relative values of these factors (scores) vary as a function of the Spanish PDO, which highlights that the composition of the vinegar categories depends also on the raw material used (wine) and on the different production methods to which the vinegars have been subjected in each PDO. Thus, these particular characteristics reflected by the scores provide the chance to discriminate the Spanish PDO corresponding to similar wine vinegar categories. In this case, related to the proven higher ability of prediction previously obtained (Table 2), only SVM classification models were built. The parameters for the optimization of the SVM models, $\log_{10}$(C) and $\log_{10}$ (γ), were found to be 2 and between −1 and 0, respectively. Table 3 summarizes the statistical results (sensitivity, specificity and errors) of the performance of the SVM models.

Regarding the "*Crianza*" category, high sensitivity and specificity values were obtained for these samples according to their origin (Spanish PDO) with classification errors of prediction lower than 3.5% (Table 3). These results demonstrate that it is possible to successful differentiate the Spanish PDO of "*Crianza*" vinegars according to their fluorescent composition that is highly related to the raw material (wine) used. Furthermore, all the samples belonging to the "*Pedro Ximenez*" category were correctly classified in the "*Vinagre de Jerez*" and "*Vinagre de Montilla-Moriles*" PDOs. The high levels of sensitivity and specificity and the good classification rates obtained were explained by the different production process employed by each Spanish PDO. "*Reserva*" was the worst classified category according to the PDOs, showing sensitivity and specificity values higher than 70% and predicted errors lower than 15%. In the case of samples belonging to "*Vinagre de*

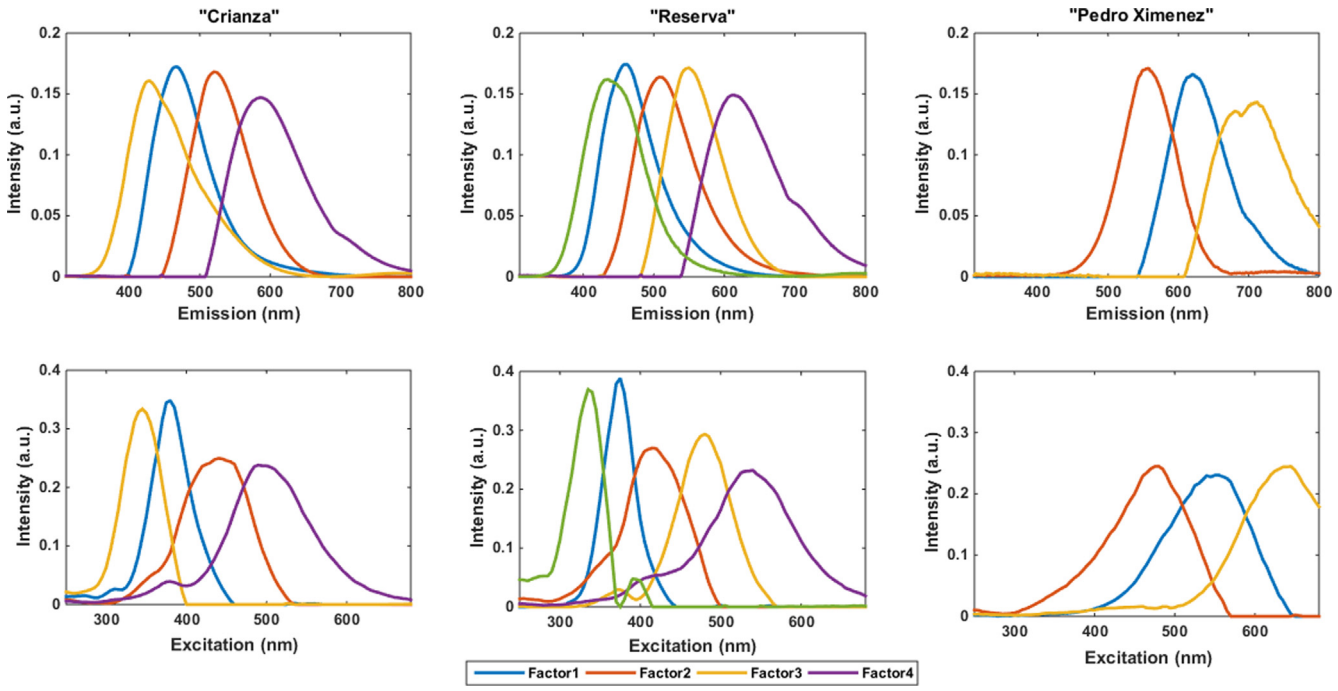

Fig. 3. Excitation and Emission spectra (PARAFAC loadings) of the main fluorophores present in different vinegars categories of the Spanish PDOs.

Table 3
Sensitivity, specificity and classification errors (%) obtained for Spanish PDO classification models in similar wine vinegar categories.

Category	CR			RE			PX	
Classification model	SVM			SVM			SVM	
Spanish PDO	JCR	MCR	CSO	JRE	MRE	CRE	JPX	MPX
Sensitivity CAL	92.9	100.0	100.0	100.0	100.0	71.4	100.0	100.0
Sensitivity CV	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Sensitivity PRED	92.9	100.0	100.0	100.0	100.0	71.4	100.0	100.0
Specificity CAL	100.0	95.0	100.0	81.8	100.0	100.0	100.0	100.0
Specificity CV	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Specificity PRED	1.00.0	95.0	100.0	1.00.0	1.00.0	0.71.4	1.00.0	1.00.0
Class. Error CAL	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Class. Error CV	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Class. Error PRED	3.5	2.5	0.0	9.1	0.0	14.3	0.0	0.0

Montilla-Moriles", 100% of the "*Reserva*" vinegars were correctly classified, and only some samples were misclassified between "*Vinagre de Jerez*" and "*Vinagre de Condado de Huelva*" PDOs (Table VIII Supplementary Material). These results are considered acceptable considering the high variability of these samples due to the wide range of aging periods that are reflected by their complex chemical composition (Casale et al., 2006; García Parrilla et al., 1999).

4. Conclusions

The analytical methodology proposed in this study, namely fluorescence excitation–emission spectroscopy coupled to PARAFAC modelling and SVM classification method, has demonstrated to be able to characterize and classify the three Spanish PDOs wine vinegars according to their Protected Designation of Origin as well as their categories (aged and sweet). As a simple preliminary characterization, a visual assessment of the fluorescence Excitation-Emission Matrices (EEMs) of the aged categories pointed out similarities in the fluorescence landscapes for the three Spanish PDOs wine vinegars: the spectral maxima were shifted towards longer wavelengths with the aging of these vinegars. Moreover, the sweet category "*Pedro Ximenez*" showed its excitation and emission maxima even at longer wavelengths than the aged categories, probably due to the different production process to which these vinegars are subjected. PARAFAC was carried out to spectroscopically and chemically characterize the different wine vinegars. It gave information about the potential fluorescent compounds present in the wine vinegars as well as their contribution in each Spanish PDO and category. These dissimilar spectroscopic and chemical features allowed us their differentiation according to their category and origin (Spanish PDO) using suitable classification methods. The feasibility of SVM methodology to classify the different categories of wine vinegars within each PDO was successfully demonstrated. The built SVM classification models proved a higher ability of prediction (between 92% and 100% correctly classified samples) than PLS-DA models, especially for classifying aged vinegar categories with similar spectroscopic characteristics. Furthermore, SVM models were also able to differentiate the Spanish PDOs even for similar vinegar categories due to their spectral differences.

The advantages of this methodology, e.g. fast, non-destructive and non- sample preparation, would allow implementing this method as an alternative tool for PDO regulatory councils and producers to be implemented in routine analysis. It could be applied for assessing the authenticity of the Spanish PDO and the vinegar

category. Finally, it is expected that further information about the specific aging periods to which these vinegars are subjected will improve the performance of some classification models.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2017.02.118.

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SUPPLEMENTARY MATERIAL

Table I. Spanish PDOs wine vinegars.

Spanish PDO	**"*Vinagre de Jerez*"**	**"*Vinagre de Montilla-Moriles*"**	**"*Vinagre de Condado de Huelva*"**
Total production (in liters)	4.7 million in 2015	4811.0 in 2014	3.5 million in 2013
Number of companies in the PDO	40	7	10
Date of PDO registration (UE)	30/9/2011	15/01/2015	30/9/2011
Geographical Area	North of Cadiz province and south of Seville province	Southern Cordoba province	Southeastern of Huelva province
Regulatory Council*	www.vinagredejerez.org	www.motillamoriles.es	www.condadodehuelva.es

Table II. Number of samples (by triplicate) used in each dataset for developing Spanish PDOs wine vinegars classification models.

Model	**Discrimination the wine vinegar category within each Spanish PDO**											
Spanish PDO	***"Vinagre de Jerez"***				***"Vinagre de Montilla-Moriles"***				***"Vinagre de Condado de Huelva"***			
Category	**JCR**	**JRE**	**JPX**	**Total**	**MCR**	**MRE**	**MPX**	**Total**	**CSC**	**CSO**	**CRE**	**Total**
Training set	30	24	9	63	15	9	9	33	12	12	15	39
Test set	9	9	3	21	6	3	6	15	6	6	6	18
Model	**Differentiation the Spanish PDO corresponding to similar wine vinegar categories**											
Category	***"Crianza"***				***"Reserva"***				***"Pedro Ximenez"***			
Spanish PDO	**JCR**	**MCR**	**CSO**	**Total**	**JRE**	**MRE**	**CRE**	**Total**	**JPX**	**MPX**		**Total**
Training set	27	15	12	54	24	9	15	48	9	9		18
Test set	12	6	6	24	9	3	6	18	3	6		9

Table III. Quality parameters of the PARAFAC models for each Spanish PDO wine vinegar.

Spanish PDO	***"Vinagre de Jerez"***	***"Vinagre de Montilla-Moriles"***	***"Vinagre de Condado de Huelva"***
Number of Factors	5	5	4
Variance explained (%)	99.42	99.52	97.95
CORCONDIA* (%)	44.91	67.62	46.63

*CORCONDIA= CORe CONsistency DIAgnostic test

Table IV. Fluorescence excitation and emission maxima (nm) of the main fluorophores presented in each Spanish PDO wine vinegar

Note: *Colours are related to the factors obtained by PARAFAC models (Figure 2) in decreasing order of variance explained: blue (Factor 1), red (Factor 2), yellow (Factor 3), purple (Factor 4), green (Factor 5).*

Spanish PDO	Excitation and emission maxima (nm)				
	F1	F2	F3	F4	F5
"Vinagre de Jerez"	465/535	400/480	500/580	350/440	585/655
"Vinagre de Montilla-Moriles"	375/460	410/500	470/550	340/420	530/605
"Vinagre de Condado de Huelva"	370/470	420/505	300/425	485/560	-

Table V. Mean values (a.u.) and variances (a.u.) of the fluorophores obtained for each Spanish PDO according to the vinegar category.

Spanish PDO	Category Acronym	F1	F2	F3	F4	F5
"Vinagre de Jerez"	JCR	5.67±5.33	5.56±5.86	3.94±3.70	2.87±3.83	1.73±1.58
	JRE	3.69±1.92	2.77±2.11	2.94±0.83	0.98±0.94	1.33±0.51
	JGR	1.74±1.49	0.49±0.45	2.24±1.34	0.05±0.05	0.11±0.21
	JPX	0.37±0.25	0.10±0.08	0.86±0.63	0.01±0.01	1.13±0.48
"Vinagre de Montilla-Moriles"	MCR	19.91±9.20	11.00±4.63	6.21±3.08	6.41±4.02	1.46±0.81
	MRE	8.84±9.12	8.52±6.72	6.14±3.35	2.39±2.27	1.97±0.39
	MGR	0.93±0.03	1.79±0.09	2.63±0.20	0.42±0.01	2.26±0.17
	MPX	0.03±0.03	0.23±0.17	0.57±0.53	0.02±0.02	1.87±1.01
"Vinagre de Condado de Huelva"	CSC	20.70±17.49	3.88±2.07	7.62±9.01	1.35±0.75	
	CSO	7.02±7.31	6.08±6.05	0.76±0.81	4.17±2.51	
	CRE	5.40±3.77	5.61±3.76	0.31±0.31	5.53±2.97	
	CAN	2.66±1.38	4.99±2.77	0.00±0.00	7.50±3.17	

Table VI. Confusion matrix obtained for the SMV models built for vinegar category classification within each Spanish PDO (by triplicate).

Spanish PDO	**"Vinagre de Jerez"**			**Spanish PDO**	**"Vinagre de Montilla-Moriles"**			**Spanish PDO**	**"Vinagre de Condado de Huelva"**		
Actual class (CV)	**JCR**	**JRE**	**JPX**	**Actual class (CV)**	**MCR**	**MRE**	**MPX**	**Actual class (CV)**	**CSC**	**CSO**	**CRE**
PRED as JCR	30	0	0	PRED as MCR	15	0	0	PRED as CSC	12	0	0
PRED as JRE	0	24	0	PRED as MRE	0	9	0	PRED as CSO	0	12	0
PRED as JPX	0	0	9	PRED as MPX	0	0	9	PRED as CRE	0	0	15
Actual class (PRED)	**JCR**	**JRE**	**JPX**	**Actual class (PRED)**	**MCR**	**MRE**	**MPX**	**Actual class (PRED)**	**CSC**	**CSO**	**CRE**
PRED as JCR	9	0	0	PRED as MCR	6	0	0	PRED as CSC	3	0	0
PRED as JRE	0	9	0	PRED as MRE	0	3	0	PRED as CSO	3	6	0
PRED as JPX	0	0	3	PRED as MPX	0	0	6	PRED as CRE	0	0	6

Table VII. Quality parameters of the PARAFAC models for each wine vinegar category.

Vinegar category	***"Crianza"***	***"Reserva"***	***"Pedro Ximenez"***
Number of Factors	4	5	3
Variance explained (%)	98.77	99.45	97.48
CORCONDIA* (%)	75.35	42.91	92.36

*CORCONDIA= CORe CONsistency DIAgnostic test

Table VIII. Confusion matrix obtained for the SMV models built for Spanish PDO classification of similar wine vinegar categories (by triplicate).

Category	***"Crianza"***			**Category**	***"Reserva"***			**Category**	***"Pedro Ximenez"***	
Actual class (CV)	**JCR**	**MCR**	**CSO**	**Actual class (CV)**	**JRE**	**MRE**	**CRE**	**Actual class (CV)**	**JPX**	**MPX**
PRED as JCR	27	0	0	PRED as JRE	24	0	0	PRED as JPX	9	0
PRED as MCR	0	15	0	PRED as MRE	0	9	0	PRED as MPX	0	9
PRED as CSO	0	0	12	PRED as CRE	0	0	15			
Actual class (PRED)	**JCR**	**MCR**	**CSO**	**Actual class (PRED)**	**JRE**	**MRE**	**CRE**	**Actual class (PRED)**	**JPX**	**MPX**
PRED as JCR	12	0	0	PRED as JRE	9	0	3	PRED as JPX	3	0
PRED as MCR	0	6	0	PRED as MRE	0	3	0	PRED as MPX	0	6
PRED as CSO	0	0	6	PRED as CRE	0	0	3			

ARTÍCULO 4

Excitation-emission fluorescence as a tool to assess the presence of grape-must caramel in PDO wine vinegars

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ABSTRACT

A practice in wine vinegar production is the addition of grape-must caramel to correct and unify the final colour of different batches. Although current legislation allows it, the effect in vinegars' quality has not been studied yet and it can become a fraud when it is used to simulate the effect of a longer ageing. Therefore, the aim of this work was to assess multidimensional fluorescence as a cost-effective and fast technique for detecting and quantifying grape-must caramel in vinegars. Different amounts of grape-must caramel and multivariate data analysis, as Parallel Factor Analysis (PARAFAC), N-way partial least squares and partial least squares discrimination and regression (NPLS-DA, PLS-DA and NPLS) were studied. Triangle sensory test was also performed. Results demonstrated the ability of this methodology in the detection and quantification of grape-must caramel (low prediction errors, RMSEP ≈ 0.24) and the effects that grape-must caramel has upon a PDO vinegar's final quality.

1. Introduction

Wine vinegar is the most commonly-used vinegar in both Mediterranean countries and Central Europe. Andalusia is a southern Spanish region traditionally associated with wine growing where three high-quality wine vinegars have been protected under a legal framework called Protected Designation of Origin (PDO): *Vinagre de Jerez*, *Vinagre de Montilla-Moriles*, and *Vinagre de Condado de Huelva* PDOs (Council Regulation (EC) No 510/2006). These high-quality PDO wine vinegars are made from the corresponding protected wines, endowing each vinegar with singular and specific characteristics. All of the PDO regulations require an ageing period in wooden butts and during this ageing period an important number of physicochemical changes take place. These changes are what give the vinegars their unique organoleptic properties and sensory quality (Morales, Tesfaye, García-Parrilla, Casas, & Troncoso, 2002). *Vinagre de Jerez* and *Vinagre de Montilla-Moriles* PDOs have established the same categories regarding sweetness, time and method of ageing (the *criaderas and solera* and *añada* system): *Pedro Ximenez* category (sweet category), *Crianza* (aged in wood for at least 6 months), *Reserva* (with a minimum ageing time of 2 years.) and *Gran Reserva* (aged for 10 or more years). During ageing, the flavours of the barrel are absorbed by the vinegar and therefore, their quality increases. This fact raises the final market price, thus making them more vulnerable to frauds (Callejón et al., 2012). This means that PDO wine vinegar quality assurance and authentication are highly important issues.

Authenticating and characterising PDO-labelled vinegars with the aim of assuring their quality, is important for protecting the consumer against being sold an inferior quality or counterfeit product (Danezis, Tsagkaris, Camin, Brusic, & Georgiou, 2016; Karoui & De Baerdemaeker, 2007). The unfair activities related to high-quality wine vinegars that bear a PDO label range from incorrect labelling to production outside PDO regulations or even to adding substances prohibited by the regulations. One of the substances added to the vinegars is grape-must caramel.

Grape-must caramel, also called 'grape syrup', is a sweetening and colouring agent obtained after boiling the grape must which is very rich in sugars and is brown in colour (Ortega-Heras & González-Sanjosé, 2009). It is commonly added to some Spanish wines in order to obtain special sweet wines. The addition of grape-must caramel to Spanish PDO wine vinegars is an allowed practice performed to unify the final colour of vinegars of different batches. The amounts required for this

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purpose are low and they should not affect the organoleptic characteristics of the final products. However, due to the fact that a maximum limit of addition has not yet been established, this could lead to some adulterations with the aim of modifying some of the characteristics of the final wine vinegar.

During ageing the colour of wine vinegar changes from amber to mahogany. The content and concentration of polyphenols, tannins and anthocyanins as well as an oxidation process are the main factors involved in the vinegar's darkening. Many of these compounds are also present in grape-must caramel, making determination of the presence of grape-must caramel in vinegars a difficult issue. In this context, the addition of grape-must caramel to the final wine vinegars could be used to simulate the effect of a greater wood ageing in wine vinegars. It has been demonstrated that the addition of grape-must caramel to a wine vinegar produces significant changes in its composition and final characteristics with a large increase in both brown tonalities and sweetness (Ortega-Heras & González-Sanjosé, 2009). Thus, the addition of grape-must caramel to a vinegar could change its organoleptic characteristics, the final product being different from the raw one. All of these facts illustrate the need for an analytical tool to determine and monitor the addition of grape-must caramel to PDO-protected wine vinegars.

In recent years, interest has been growing in developing rapid, inexpensive, non-destructive and direct methodologies based on non-targeted techniques for food characterisation. In this context, today excitation-emission fluorescence spectroscopy has an important role. Among the advantages of fluorescence spectroscopy is the enhanced selectivity when compared to other spectroscopic techniques; its high sensitivity to a wide range of potential analytes and an easy – or even unnecessary – sample pre-treatment (Sayago, García-Gonzalez, Morales, & Aparicio, 2007). Fluorescence spectroscopy has been applied as a competitive, high sensitivity, fast and non-destructive technique in food analysis (Karoui & Blecker, 2011). In a previous study (Ríos-Reina et al., 2017) this methodology demonstrated its usefulness for characterising and classifying PDO wine vinegars

Measuring the emission spectra at different excitation wavelengths results in a three-dimensional Excitation-Emission Matrix (EEM) array, which contains information unique to each measured sample. Nowadays, the instrumental improvements and the availability of software specially designed to extract information contained in spectra has enabled the use of EEM in combination with chemometric methods in order to characterize and detect adulteration in different matrices, such as different food products and beverages (Azcarate, Teglia, Karp, Camiña, & Goicoechea, 2017; Casale et al., 2018; Elcoroaristizabal et al., 2016; Öztürk, Ankan, & Özdemir, 2010; Sayago et al., 2007), as well as in many other matrices (Heidari, Hemmateenejad, Yousefinejad, & Moosavi-Movahedi, 2018; Zhu et al., 2016). The analytical information contained in fluorescence spectra can be extracted in order better to interpret it using various multivariate analysis techniques that relate several analytical variables to the analytes' properties. One appropriate multiway method for extracting and interpreting the maximum information possible from this matrix is PARAllel FACtor Analysis (PARAFAC). It has been applied in order to break fluorescence EEMs down into different independent groups of fluorophores, as well as their relative concentration (scores) in each sample (Bro, 1997). The information provided by the resolved fluorophores has been successfully applied in food quality control since it can reveal clearer insights into the relationships between the intrinsic food properties and the quality of the product. Moreover, the extracted fluorophores could be used for a classification approach by discriminant analytical methods such as partial least squares-discriminant analysis (PLS-DA). In addition, the EEM array could also be studied directly with the use of multivariate calibration methods such as N-way partial least squares (N-PLS) that have also made it possible to relate instrument responses that consist of several variables to a chemical or physical property of a sample, as well as with multiway discrimination analysis such as NPLS-DA.

The aim of this study was to assess the potential of excitation-emission fluorescence spectroscopy combined with three-way methods of analysis (PARAFAC and multiway N-PLS regression) and discriminant analysis (PLS-DA and NPLS-DA) to detect and classify the different additions of grape-must caramel in PDO wine vinegars. It is the first time that a methodology for the determination of gape-must caramel has been established. Different amounts of grape-must caramel were added to PDO wine vinegars that were grape-must caramel free in their raw composition. In addition, commercial PDO wine vinegars (that actually could have some added grape-must caramel) were also analysed to test the models and to determine their amount of caramel. For this purpose, Parallel Factor analysis (PARAFAC) was applied for pre-processing the three-dimensional arrays in order to study the potential fluorophores related to this addition. Multivariate data analysis (PCA, PLS-DA) was then performed in order to differentiate and classify samples that had or did not have grape-must caramel in different concentrations. Consequently, the discrimination results were compared to those obtained by a multiway partial least-squares discrimination analysis (NPLS-DA). Finally, regression models were developed in order to attempt to predict and quantify the level of grape-must addition by relating the PARAFAC components to the chromatographic compounds detected, or by using the EEM array by N-PLS regression method. Additionally, a sensory test was developed to evaluate the influence of added grape-must caramel on the organoleptic properties of the PDO wine vinegars and to propose a possible addition limit that does not affect or modify their unique final organoleptic properties.

2. Materials and methods

2.1. Samples

Wine vinegar samples from two Spanish PDOs (*Vinagre de Jerez* and *Vinagre de Montilla-Moriles*) were analysed in this study: 16 commercial wine vinegars from the *Crianza* category (CR), aged for 6 months to 2 years (10 from *Vinagre de Jerez* PDO and 6 from *Vinagre de Montilla-Moriles* PDO) and 18 commercial wine vinegars from the *Reserva* category (RE), aged from 2 to 10 years (13 from *Vinagre de Jerez* PDO and 5 from *Vinagre de Montilla-Moriles* PDO). These samples were collected working in compliance with the Regulatory Councils and were grouped in this study as the Unmodified group. Finally, 2 caramel-free samples of both *Crianza* and *Reserva* (one from each PDO) were collected from the wineries and included in the study as Control samples. More information and codification of samples is shown in Table 1.

2.2. Reagents and chemicals

The grape-must caramel (also named colourant caramel MO-7) used was supplied by SECNA S.A. (Valencia, Spain), with identification number CEE: E − 150 d. Water was obtained from Milli-Q purification system (Millipore, USA). Analytical-quality acetic acid and methanol were supplied by Merck (Darmstadt, Germany). 5-Hydroxymethylfurfural (5-HMF) according to the standard OIV (2009) method was purchased from Sigma–Aldrich (Madrid, Spain).

2.3. Grape-must caramel addition

First, thirteen different amounts of a dilution of grape-must caramel (10/100 v/v) were added to 10 mL of vinegar: 5, 10, 20, 30, 40, 50, 75, 100, 125, 150, 175, 200, and 250 μL. The amounts added were selected by examining the total range of colours of the commercial wine vinegars. These samples were grouped into a class called Modified. The vinegars selected as a matrix of these different additions were the *Crianza* and *Reserva* vinegars without caramel in their composition collected directly from the winery and belonging to both PDOs were designated as the Control samples. In Table 1, therefore, these samples

Table 1
Samples included in the study.

Class		Unmodified		Modified (curves made by addition of grape-must caramel)		TOTAL
		Control samples (without caramel)	Commercial samples (possibility of having caramel)	Control matrix (0.05, 0.10, 0.20, 0.30, 0.40, 0.50, 0.75, 1.00, 1.25, 1.50, 1.75, 2.00, 2.50% v/v)	Commercial matrix (0.05, 0.10, 0.30, 0.50, 1.00, 1.50, 2.00, 2.50% v/v)	
"Vinagre de Jerez"	Crianza (JCR)	1 (JCCR)	10 (JCR)	13	8	32
	Reserva (JRE)	1 (JCRE)	13 (JRE)	13	–	27
"Vinagre de Montilla-Moriles"	Crianza (MCR)	1 (MCCR)	6 (MCR)	13	8	28
	Reserva (MRE)	1 (MCRE)	5 (MRE)	13	–	19
6% Hydroacetic matrix (HA)		–	–	6 (0.10, 0.25, 0.50, 1.00, 1.50, 2.00%)		6
Total		*38*		*74*		**112**

appear in the Modified-control matrix group. Moreover, among these samples made, five, with intermediate concentrations of grape-must caramel (20, 40, 75, 125, 175 μL), were used as the test set for assessing the robustness of the regression models. These additions are expressed in Table 1 as % v/v.

In addition, and in order to include more samples in the models, the same procedure was performed using a commercial *Crianza*-category wine vinegar from each PDO (also grouped as Modified samples) by making 8 points of the above mentioned (group of samples named in the study as Modified-Commercial matrix). Two replicates per level were performed. A total of six curves were obtained by varying the matrix where the grape-must caramel was added: 4 *Crianza* (two control and two commercial matrices) and 2 *Reserva* wine vinegars (control matrices). This information is more easily shown schematically in Table 1.

Finally, the same calibration levels were performed in a hydroacetic matrix at 6% in order to study the pure grape-must caramel. A schema and some photos of these curves are shown in Supplementary Fig. 1.

2.4. Fluorescence analysis

Fluorescence measurements were recorded using a Varian Cary-Eclipse fluorescence spectrophotometer (Varian Iberica, Madrid, Spain), equipped with two Czerny-Turner monochromators, and a Xenon discharge lamp pulsed at 80 Hz with a half peak height of 2 ms (peak power equivalent to 75 kW). A high-performance R298 photomultiplier tube detector was used for collecting the fluorescence spectra. Wine vinegar samples were analysed directly without sample pre-treatment by pipetting them into 3.5 mL quartz cuvettes before measurement. 1-cm path length standard quartz cells (Hellma Analytics, Müllheim, Germany) were used to perform the measurements in a Peltier thermostatic cuvette holder (25.00 ± 0.05 °C). The spectrometer was interfaced to a computer with Cary-Eclipse software for spectral acquisition and exportation.

The fluorescence Excitation-Emission Matrices (EEMs) were obtained by varying the excitation wavelength (λ_{ex}) ranging between 250 and 650 nm (every 5 nm), and recording the emission spectra (λ_{em}) from 300 to 700 nm (every 4 nm). For these measurements, excitation and emission slits were both set at 5 nm, and the scan rate was fixed to 1200 nm min^{-1}. The system was wavelength-calibrated every day by means of the water Raman peak to account for a possible instrument wavelength drift. EEMs were recorded in triplicate for each wine vinegar type and each level of the calibration and pre-processed in order to avoid noisy and non-informative areas by selecting shorter spectral ranges (λ_{ex} from 300 to 650 nm, and λ_{em} from 300 to 700 nm).

2.5. High-performance liquid chromatography (HPLC) analysis

HPLC analysis was performed using a LaChrom® WWR-Hitachi (Barcelona, Spain) liquid chromatograph with a quaternary L-7100 pump connected to an L-7455 diode array detector (DAD). The column was a Luna C18, 5 μm, 250 × 4.6 mm and a guard precolumn of 4.0 × 3.0 mm from Analytical Phenomenex (Torrance, CA, USA). Detection was performed at 280 nm. The injection of the samples (10) μL was performed using an L-2200 autosampler and the separation was obtained at a flow rate of 1.2 mL min^{-1} with an isocratic elution. The analysis takes less than five minutes.

The mobile phase consisted of 80% water, 18% methanol and 2% acetic acid. Previously filtered through a 0.45 μm PTFE membrane filter (Merck, Darmstadt, Germany), the samples were analysed in duplicate. Quantification of 5-HMF was performed according to Elcoroaristizabal et al. (2016), by using an external calibration curve in the range between 5 and 80 ppm. A calibration curve at 6 levels with two replicates per level was built using the least-squares method. The response of the 5-HMF standard was linear within the concentration range tested, with a determination coefficient of $R^2 = 0.997$. Standard solutions were

prepared using a hydro-acetic matrix (6% v/v).

2.6. Sensory analysis

An olfactory and taste analysis was carried out. The expert sensory panel comprised eight tasters (six females and two male), all belonging to our laboratory and with extensive experience in wine vinegar sensory analysis. For the olfactory test, fifteen millilitres of each sample were presented in coded opaque glasses to mask the colour while following the protocol for vinegars established by Tesfaye et al. (2010). For the gustative test, a drop of each sample was placed in a coffee spoon.

Firstly, an ascending order test was performed to delimit the correct concentration range of grape-must caramel to study and to familiarize panellists with the odour of the samples. Panellists were asked to indicate in which glass and spoon they perceived any change of odour or flavour. The starting point was the CR control without any caramel. Secondly, triangular tests (IS0 4120-1983) were performed to ascertain whether the panellists were capable of discriminating caramel-free samples from those vinegars with added grape-must caramel. Moreover, triangle tests were also performed to assess the capability of discriminating some *Reserva* commercial wine vinegars from the modified wine vinegars from each PDO.

2.7. Software and data analysis

2.7.1. Pre-processing of spectra and PARAFAC analysis

EEMs data were pre-processed in order to correct Rayleigh and Raman scattering (Elcoroaristizabal, Bro, García, & Alonso, 2015) by removing and replacing the scattering areas with interpolated values by using the FLUCUT function included in the PLS_Toolbox. The corrected EEM matrices underwent PARAlell FACtor analysis (PARAFAC) (Bro, 1998) in order to extract the relevant information and to develop models for differentiating authentic samples from those with added grape-must caramel. This methodology is not described here due to having been described in a previous study (Ríos-Reina et al., 2017). The number of factors for each model was determined by using the CORe CONsistency DIAgnostic test (COR-CONDIA) (Bro & Kiers, 2003), the model percentage of explained variance and by visual inspection of the recovered spectral profiles and residuals. Non-negative constraints for all modes were applied.

2.7.2. Exploratory and classification analysis

2.7.2.1. PCA and PLS-DA on the PARAFAC factors. In order to perform a first screening of samples and to reflect the sample distribution in latent space, principal component analysis (PCA) was applied to the scores of the PARAFAC factors obtained. Moreover, classification accuracy was calculated by means of Partial Least Squares-Discriminant Analysis (PLS-DA). This algorithm was used to build classification models for discriminating the Unmodified (commercial) wine vinegar samples from the Modified samples, that is, those CR and RE with the addition of grape-must caramel and the control ones, in order to test the ability of the methodology to discriminate between the presence or absence of grape-must caramel at different levels. Furthermore, the data was autoscaled and samples were randomly divided into the training set (comprising 75% of samples) that was used for data modelling and internal validation by means of a venetian blinds cross-validation, and a test or prediction set used for evaluating the discriminative power of the models (external validation).

2.7.2.2. N-PLS discriminant analysis (NPLS-DA). NPLS-DA was applied to the three-dimensional array, which was prior multiway centred, in order to compare the classification results of a multiway analysis to the previous one-way approach (i.e. PLS-DA classification by the use of the PARAFAC factors). NPLS-DA is an extension of PLS, used in the case of data in three-dimensional arrays. Thus, the NPLS-DA consists of applying the N-PLS algorithm to classification, predicting the membership of a sample to a qualitative group defined as a preliminary (Vigneau, Qannari, Jaillais, Mazerolles, & Bertrand, 2006). In essence, N-PLS for discriminant analysis is the same as for calibration purposes. Discrimination quality was obtained by comparing the predicted groups to the real groups and is shown as the percentage of correct classification. The data was again autoscaled and randomly divided again into two sample sets, as had been the case with the PLS-DA model: the training set (comprising 75% of the samples) that was used for calibration and internal validation of the models by means of a venetian blinds cross-validation, and a test set used for evaluating the discriminative power of the models employed as an external validation.

2.7.3. Correlation of wine vinegars EEM spectra with grape-must caramel

Regression models based on PARAFAC and N-PLS algorithms were compared. On the one hand, the area of the compounds detected by HPLC as well as the % v/v of grape must-caramel were correlated to the extracted PARAFAC components. On the other hand, a multiway linear regression analysis, called N-way partial least squares (N-PLS), was built using the EEM data which was multiway centred in order to determine the presence of grape-must caramel in the commercial PDO wine vinegars by the fluorescence landscapes kept as three-way array. Regression models were evaluated using the figures of merit: Root Mean Square Error of calibration, cross-validation and prediction (RMSEC, RMSECV and RMSEP) as a term to indicate the prediction error of the model, and the coefficient of determination (R^2). R^2, generally used for evaluating model quality, is the correlation coefficient between the predicted and actual/measured grape-must caramel. RMSEC is used to compare quality of the results provided in the calibrations and it is expressed as a percentage (in both calibration and prediction), taking into account the response range in its calculation (Sáiz-Abajo, González-Sáiz, & Pizarro, 2006). The data was multiway centred across the first mode (i.e. sample mode) and divided into two sets, train and test. Venetian blinds was applied by means of cross validation.

2.7.4. Software

EEM data modelling and chemometric analyses were performed by using the PLS_Toolbox 7.9.5 (Eigenvector Research Inc., Wenatchee, WA) working under Matlab v.8.5.0 environment (The Mathworks Inc., Natick, MA).

3. Results and discussion

3.1. Visual assessment of fluorescence landscapes

Fig. 1 shows, in the left side (a), an example of the fluorescence landscapes in the form of contour plots (after removing and replacing the scattering areas) of different levels of the calibration curve made with the *Crianza* Control wine vinegars as matrix (those without caramel obtained from the wineries) from both PDOs, including also the *Reserva* Control wine vinegars on the far right of the figure (Fig. 1a). Moreover, the calibration curve produced with the hydroacetic matrix is also shown at the left bottom of the figure (Fig. 1c).

As can be observed, a visual assessment of the fluorescence landscapes indicated a similar profile for vinegars of both PDOs, with fluorophores overlapping in both excitation and emission dimensions, together with some differences due to the addition of grape-must caramel. Thus, the fluorescence profiles of the *Crianza* vinegars without grape-must caramel (first samples in the rows) showed a common maximum peak around 370/450 nm for both excitation/emission wavelengths ($\lambda_{ex}/\lambda_{em}$), although in the *Reserva* control samples (last samples in the rows) the maximum peaks appeared at slightly higher wavelengths, around 370–470 nm of λ_{ex} and 470–550 nm of λ_{em}. These features were similar to those observed in a previous work studying PDO wine vinegars (Ríos-Reina et al., 2017).

Additionally, the visual assessment of the EEM landscapes with and

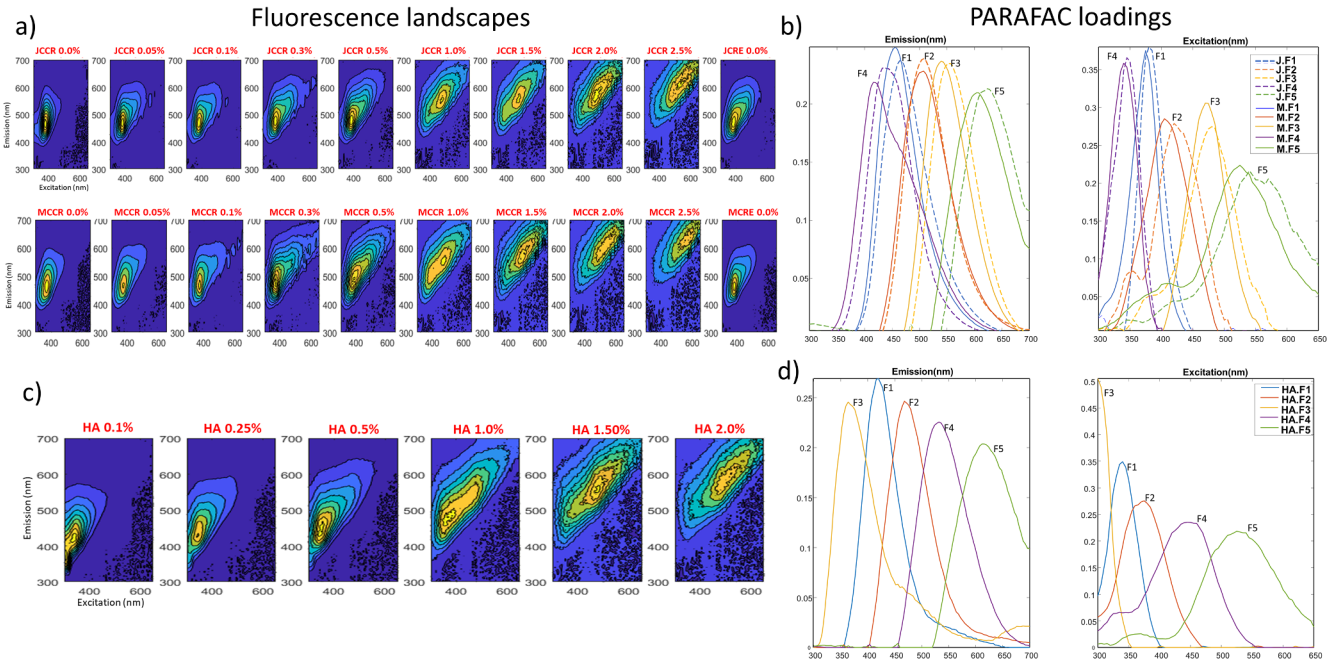

Fig. 1. Fluorescence landscapes in the form of contour plots and PARAFAC loadings (excitation/emission profiles) of each main fluorophore of different sets of samples: Calibration curves made with the Crianza "Control" wine vinegars as matrix from both PDOs (a); All the samples from both PDOs (Modified and Unmodified) (b); Grape-must caramel calibration curve made with the hydroacetic matrix ((c) and (d)).

without the addition of grape-must caramel allows an a *priori* confirmation of differences between samples by looking at the areas where the potential compounds appeared. Thus, for example, the peak at 370/450 nm ($\lambda_{ex}/\lambda_{em}$) tended to disappear as more grape-must caramel was added, giving way to the appearance of a second peak around 550/570 nm of excitation and emission wavelength, respectively. It should be also noticed that, as the commercial samples are able to present some grape-must caramel, some of the analyzed in this study already showed this trend. Moreover, another important feature was that as more grape-must caramel was added to the vinegar, EEM intensity decreased. This behaviour was also observed as being PDO-independent – even in the hydroacetic matrix analysed (Fig. 1c). In fact, the hydroacetic samples with different amounts of grape-must caramel showed similar trends, also being similar to the vinegar samples due to the fact that it should be considered that grape-must caramel has many grape-derived compounds, such as wine vinegars. However, the excitation/emission wavelengths were not exactly the same, due to the relevant phenomena related to the nature of the food and its molecular environment, both of which influence the fluorescence signal. This is commonly called the matrix effect (Azcarate et al., 2017). All of these results partially demonstrated that excitation-emission fluorescence was able to detect those samples whose colour was modified by the addition of grape-must caramel.

3.2. Decomposition of the spectral data in the potential fluorophores by using PARAFAC

In order to observe and evaluate the pure spectra of fluorophores related to the addition of grape-must to wine vinegars, an adequate multiway method for pre-processing the three-dimensional array was carried out. Thus, the EEM landscapes of all of the samples under study (the Modified and the Unmodified samples of both categories and both PDOs) were decomposed into the main fluorescence contributions by using PARAFAC analysis. The best PARAFAC model built for each PDO was obtained with five factors, giving final reliable models that explain more than 99% of the variance and with a core consistency over zero (Supplementary Fig. 2). Fig. 1 also shows in the right side the PARAFAC loadings (excitation/emission profiles) of each main fluorophore obtained for both PDOs (Fig. 1b) and hydroacetic matrix with different amounts of grape-must caramel (Fig. 1d). A great similarity of the spectral profiles acquired for both PDOs (*Vinagre de Jerez* in discontinuous lines and *Vinagre de Montilla-Moriles* in continuous lines) could be observed. This fact suggests that these fluorescence fingerprints could be useful for addressing the problem under study, as it shown to be PDO-independent. Similar results were obtained by Elcoroaristizabal et al. (2016) in the study of different types of *Cava* in which a great similarity of the spectral profiles was obtained independently of the *Cava* analysed.

The fluorescent loading patterns of the modelled factors in the PDO samples can be matched to fluorophores described in the literature. The first factor (F1, blue in Fig. 1b) therefore, has a similar profile for the two PDOs under study with excitation and emission maxima centred around 380 nm and 450 nm, respectively. This factor also appeared in the previous study (Ríos-Reina et al., 2017) and was related to the cumarins, tannins, phenols, flavonols that are naturally present in wine.

The second factor (F2, red in Fig. 1b) is a peak centred at 400–430 nm of excitation and 500–520 nm of emission. This fluorophore could be matched with Maillard compounds according to Zhu, Ji, Eum, and Zude (2009) and Ríos-Reina et al. (2017), formed in vinegars during ageing (García Parrilla, Heredia, & Troncoso, 1999). According to the literature, within these compounds, 5-HMF is one that has been shown to have a high correlation to these wavelengths (Callejón et al., 2012). Grape-must caramel also has high amounts of this compound. In this regard, it is important to emphasize that each PARAFAC factor probably corresponds to a related fluorescent molecule group, and not necessarily to a single fluorescent molecule and for that reason, this factor could be matched with different compounds, although from a similar family.

The third factor (F3, yellow in Fig. 1b) shows an excitation maximum around 470 and the emission one at 550 nm for both PDOs although for *Vinagre de Jerez* this factor shows a shoulder at 350 nm of excitation that could be due to differences in the composition between the two PDOs. According to the literature (Airado-Rodríguez, Durán-Merás, Galeano-Díaz, & Wold, 2011) and our previous knowledge (Ríos-

Reina et al., 2017), the common parts of this factor appeared to be related to vitamin B2 and its principal forms such as Riboflavin, Flavin mononucleotide (FMN), and Flavin adenine dinucleotide (FAD)

The fourth factor (F4, purple in Fig. 1b) has excitation and emission maxima between 320 and 340 nm and 400–420 nm, respectively. In this case, the *Vinagre de Montilla-Moriles* factor shows a small shoulder at 450 nm of emission, different to the other PDO. According to the results presented in the literature, excitation/emission wavelengths around 330/420 nm have been related to phenolic acids and phenolic aldehydes, as well as oxidation and Maillard reaction products (present due to browning processes and oxidative mechanisms taking place during ageing and storage) (Airado-Rodríguez et al., 2011; Azcarate et al., 2015; Callejón et al., 2012; Dufour, Letort, Laguet, Lebecque, & Serra, 2006; Elcoroaristizabal et al., 2016; Sádecká & Tóthová, 2007).

Finally, the fifth factor (F5, green in Fig. 1b) shows a peak centred at 550 nm of excitation, with a shoulder at 400 nm in both PDOs, and an emission maximum around 600–630 nm. This has not previously been associated to any fluorophore. However, this factor was similar to the one obtained in the previous work (Ríos-Reina et al., 2017), which showed a relationship to *Pedro Ximenez* wine vinegars. Consequently, higher mean values of this factor were obtained for samples belonging to this category. The sweet category is produced by adding raisined *Pedro Ximenez* grape must or adding *Pedro Ximenez* wine to the vinegar. Therefore, the concentration of grape-must should be higher in these sweet vinegars than in the *Crianza* or *Reserva* ones. For this reason, the presence of this factor in our samples also appeared to be related to the addition of grape-must caramel, it being, therefore, a relevant factor to take into account in this study.

As mentioned earlier, it is relevant to consider the phenomena related to the nature of the food that will influence the fluorescence signal. These phenomena are related to the inherent fluorophores' concentration and their environment. Therefore, a specific fluorophore studied in different foods can present different spectral signals (Azcarate et al., 2017). In fact, adding grape-must caramel changes the environment of the natural wine vinegar fluorophores and so could have the ability to modify the signal, as can also be observed in the 5-factor PARAFAC model of the hydroacetic matrix with only grape-must caramel in its composition (Fig. 1d). Thus, the PARAFAC model built with the curve of grape-must caramel in a hydroacetic matrix (Fig. 1d), shows similar fluorophores as in the vinegar matrix, but some of them are displaced. In spite of this, the fifth factor (F5 in green, Fig. 1.d) matched perfectly in terms of excitation/emission wavelengths with the fifth factor of the PARAFAC models developed with the PDO wine vinegars, which appeared to have a strong relationship with the presence of grape-must caramel.

In fact, only the scores of the fifth PARAFAC factor (F5) extracted from the hydroacetic curve showed an increase in the case of added grape-must caramel, appearing to follow a logarithmic kinetic (Supplementary Fig. 3). Hence, the scores of the F5 described a logarithmic kinetics equation as follows:

$$Y = m\mathrm{Ln}(Y_0) + b;$$

where Y is the score value of F5 (a.u.), m is the slope, Y_0 is the initial value of F5 score (a.u.), and b the intercept. Thus, the logarithmic kinetic obtained with the fifth PARAFAC factor, which is shown in Supplementary Fig. 3), was $Y = 42.538\mathrm{Ln}(Y_0) + 148.15$.

3.3. Exploratory analysis

A principal component model was developed with all of the Modified and Unmodified samples for each PDO by using the extracted PARAFAC factors in order to explore the data and to detect grouping and outliers in each PDO. The scores and loadings plots are shown in Fig. 2. In general, a separation of both groups (modified and unmodified) could be observed in the two PCA models for both PDOs, which means that the methodology appeared to be able to detect the addition of grape-must caramel.

In the case of the *Vinagre de Montilla-Moriles* PCA model (Fig. 2a), the first component (PC1) is the main factor in the separation, explaining 69.30% of the original variance, showing a good separation of the groups, the modified samples being located on the negative side of PC1 and the unmodified on the positive side. However, it was also observed that three unmodified samples (i.e. commercial samples) were grouped closely to the modified ones, especially two RE samples located next to the samples containing the most added grape-must caramel. These results suggest that these two RE samples could have a higher amount of grape-must caramel in their composition than the other commercial samples, something that could change the raw organoleptic characteristics by binding the effect of some compounds related to ageing; or it could even be a case of unfair practice, these RE samples in fact being CR vinegars with added grape-must caramel in order for them to resemble the colour of an RE.

With regard to the Modified samples, those with the lowest amounts of grape-must caramel (lower than 0.1% v/v) were located near to some commercial samples. Thus, a commercial *Crianza* sample was observed located very close to a Modified wine vinegar in the scores plot, this modified sample being a *Crianza* Control vinegar containing 0.05% grape-must caramel. These results showed that some commercial samples could have a very low amount of grape-must caramel in their final composition. In terms of the loadings plot, and due to its position on the plot, the fifth factor once again appeared to be the greatest factor regarding the presence of grape-must caramel, followed by F4.

With regards to the PCA model of *Vinagre de Jerez* (Fig. 2b), the separation in this particular case appeared to be more related to PC3. Thus, observing the scores plot of PC1 vs PC3, modified samples were located on the negative side of PC3, although once again, a few unmodified samples (some CR and RE commercial samples) were not properly separated from the modified ones in this model. As before, this placement could be explained by a greater amount of grape-must caramel in their composition than the rest of samples, thus affecting the composition by binding some relevant compounds. These wrongly-placed RE commercial samples therefore appeared to have more similarities according to their scores with the RE samples modified with 1–2.5% v/v of grape must caramel, as well as the fact that the aforementioned wrongly-placed CR commercial samples appeared to be more similar to the CR samples modified with 1.5–2% v/v of grape-must caramel.

The separation of both groups of samples was again explained by the F5, as could be observed in the loadings plot. However, when observing the loadings plot, F4 and F1 also appeared to play an important role in this separation. This partially agrees with the results mentioned above (Section 3.1) in which F4 was related to Maillard reaction products that could be derived from the grape-must caramel.

3.4. Classification analysis of modified (by adding grape-must caramel) and unmodified samples (commercial wine vinegars)

Once the ability of the multidimensional fluorescence spectroscopy in distinguishing the presence of grape-must caramel at different levels was demonstrated, the next step was to gain an insight into this differentiation and to determine if the extracted PARAFAC fluorophores allows the classification of samples according to the modification of vinegars with grape-must caramel. To this end, PLSDA classification models were performed using the extracted PARAFAC factors. Moreover, in order to consider the contribution of multiple effects and not only the most relevant information (PARAFAC factors), NPLS-DA classification models were also performed, taking the multiway arrays (EEMs) into consideration. Both classification models were therefore studied and compared in the following section. Prior to the classification analysis, the data set was randomly partitioned into two sets, train and test, and all of the datasets were mean-centred before developing the models.

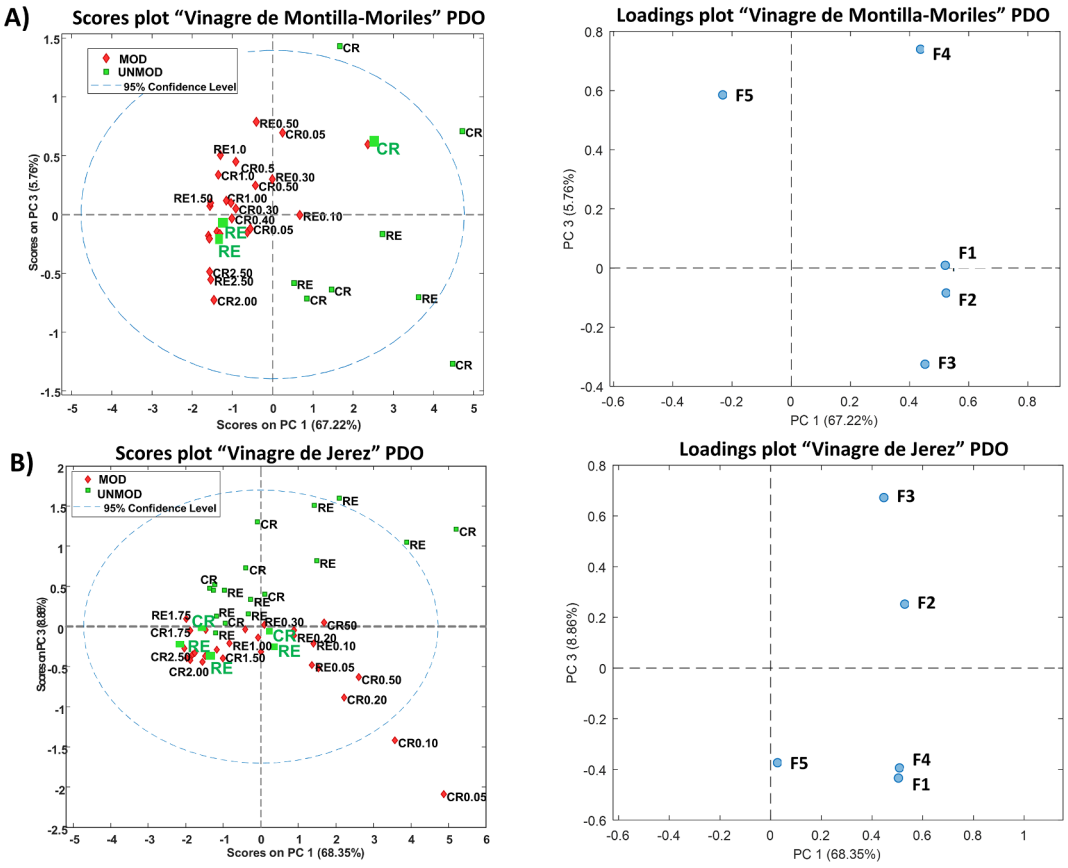

Fig. 2. Score and loading plots of the principal components obtained by a PCA by using the extracted PARAFAC factors with all the Modified (MOD) and Unmodified (UNMOD) samples: for "Vinagre de Montilla-Moriles" PDO (a); for "Vinagre de Jerez" PDO (b).

3.4.1. PLS-DA classification between modified and unmodified wine vinegars using the extracted PARAFAC factors

Two PLS-DA models were developed according to each PDO including samples from the two groups in the train and test sets. The *Vinagre de Jerez* PLS-DA model was obtained using 4 latent variables (LVs), which explained 99.75% of total variance, while the PLS-DA model of *Vinagre de Montilla-Moriles* was obtained using 3 LVs and explained 96.83% of total variance. Table 2 shows the PLS-DA classification results expressed as the percentage of correct classification and the number of samples misclassified for each class. Additionally, the statistical performance parameters of the classification models (i.e. sensitivity, specificity and classification error of calibration (CAL), cross-validation (CV) and prediction (PRED)) are shown in Supplementary Table 1. Correct classification rates of 100% were obtained for both Modified and Unmodified groups in the training set for each PDO. In this way it was observed that the models were able to classify the unmodified samples, where both CR and RE commercial samples are grouped, from those modified with the addition of grape-must caramel. To test the models, those commercial samples that were not well-located on the previous exploratory models were purposely included in the prediction sets, together with other unmodified and modified samples in order not to disturb the model's calibration. The classification results enabled the results observed by the previous PCA models to be confirmed, since the seven misclassified samples were those that behaved differently to the rest of commercial PDO wine vinegars.

Moreover, the classification results showed that a 100% correct classification was achieved for all of the modified samples for the prediction set, confirming the good predictive ability of the classification models developed and, hence, multidimensional fluorescence spectroscopy's ability to detect the addition of grape-must caramel to wine vinegars.

Furthermore, the possibility of taking both PDOs into account together was tested. Table 2 shows that the PLS-DA model obtained with 5 latent variables and 99.64% of total variance explained, again classified the same seven unmodified samples as modified wine vinegars. However, in spite of the fluorescent components appearing to be very similar in both PDOs, when a classification is performed by including both PDOs together, the percentage of correct sample classification was lower than in the separated models.

3.4.2. NPLS-DA classification between modified and unmodified wine vinegars using the three-dimensional arrays EEM

Once again it should be emphasised that each factor probably does not necessarily correspond to a single fluorescent molecule (Elcoroaristizabal et al., 2016). It is, therefore, possible that different factors need to contribute in order to explain a group of compounds. For this reason, a multiway classification approach was studied. In this case the three-dimensional arrays (EEMs) were used, NPLS-DA was performed and their results were compared to those obtained by PLS-DA with the PARAFAC factors. NPLS-DA classification results are also shown in Table 2. In addition, the statistical performance parameters of the NPLS-DA classification models are shown in Supplementary Table 1. It can be seen that a highly discriminant NPLS-DA model was obtained by using three PLS factors for both *Vinagre de Jerez* and *Vinagre de Montilla-Moriles* models. Here, and similar to the previous PLS-DA results, six commercial samples (three of *Vinagre de Jerez* and three of *Vinagre de Montilla-Moriles*) were classified as being modified with grape-must caramel. Moreover, the number of latent variables needed to explain the classification, the percentage of total variance explained and the samples misclassified (Table 2), as well as sensitivity and specificity (Supplementary Table 1), were almost the same for the

Table 2
PLS-DA and NPLS-DA classification results using the PARAFAC components and the EEMs, respectively.

PDO	LVs		% Total explained variance		Training	% Correct Classification		Samples misclassified	
	P	N	P	N		P	N	P	N
"Vinagre de Jerez"	4	3	99.7	99.7	Modified	100	100	0	0
					Unmodified	100	100	0	0
					Prediction	**% Correct Classification**		**Samples misclassified**	
						P	**N**	**P**	**N**
					Modified	100	100	0	0
					Unmodified	42.86	71.43	4 (2RE,2CR)	3 (2RE,1CR)
"Vinagre de Montilla-Moriles"	3	3	96.8	96.8	**Training**	**% Correct Classification**		**Samples misclassified**	
						P	**N**	**P**	**N**
					Modified	100	100	0	0
					Unmodified	100	100	0	0
					Prediction	**% Correct Classification**		**Samples misclassified**	
						P	**N**	**P**	**N**
					Modified	100	100	0	0
					Unmodified	25	40.00	3 (2RE,1CR)	3 (2RE,1CR)
Both PDOs together	5	3	99.6	99.1	**Training**	**% Correct Classification**		**Samples misclassified**	
						P	**N**	**P**	**N**
					Modified	90.70	89.47	4 (M < 0.75%)	4 (M < 0.75%)
					Unmodified	86.36	90.90	3 (2JRE,1JCR)	2 (1JRE,1JCR)
					Prediction	**% Correct Classification**		**Samples misclassified**	
						P	**N**	**P**	**N**
					Modified	100	100	0	0
					Unmodified	36.36	58.33	7 (4 J, 3 M)	5 (3 J,2M)

*Note: P = PLS-DA model; N = NPLS-DA model. LVs = Latent variables.

previously-discussed PLS-DA and the NPLS-DA models. As a result, both approaches could be good options to consider. This could demonstrate that the fluorophores extracted by PARAFAC were sufficient to explain the grape-must caramel effect. However, although the multiway classification approach is faster and easier to develop than undertaking PARAFAC and a PLS-DA, it provides less information with respect to the fluorophores involved.

With regard to the model considering both PDOs together and obtained by 3 LVs, better classification rates could be observed (higher percentage of correct classification and less latent variables needed) for NPLS-DA than for the model obtained by PLS-DA and PARAFAC factors, although, once again, the same seven commercial samples were misclassified. This could be explained by the fact that in the multiway discrimination methodology the whole fluorescence matrix is considered. This enables all of the fluorophores related to caramel and to the effect of its environment to be modulated, as well as being able to modulate the interferences.

3.5. Correlation between the additions of grape-must caramel and EEMs

3.5.1. Univariate calibration - HPLC analysis

After confirming the changes in vinegar components observed in the EEMs with the addition of grape-must caramel, and in order to ascertain the specific compound concentrations which increase or change with such an addition, a chromatographic analysis was performed including the modified and unmodified samples, as well as the hydroacetic solution (Fig. 3). In all of these analyses, three compounds were principally observed to increase when grape-must caramel was added with the following elution order (Fig. 3a): 2.3, 2.7 and 4.2 min of retention time. The first two compounds were unidentified, whereas the last was identified by its corresponding standard as 5-hydroxymethylfurfural (5-HMF). The 5-HMF and the compound termed as unknown 2, (retention time at 2.7 min), presented in all of the samples, while unknown 1 (retention time at 2.3 min) did not present in the wine vinegar matrices which had no grape-must caramel in their raw composition (Control samples).

Some studies in the literature show that grape-must caramel has a high amount of furfural-related compounds, including which 5-HMF (Ortega-Heras & González-Sanjosé, 2009). 5-HMF is a furanic compound formed during Maillard reactions or by direct dehydration of sugars under acidic conditions (caramelisation) during thermal treatments applied to foods (Capuano & Fogliano, 2011). Hence, its concentration should be high in grape-must caramel. However, as can be observed in the calibration curves of the areas of the three compounds and in the % of grape-must caramel (Fig. 3b), the compound that presented the highest slope was the one named unknown2, and not, as expected, 5-HMF. This could be explained by the fact that other compounds have been also determined in the grape-must caramel and cooked musts, such as melanoidins, caramels (formed by non-enzymatic browning reactions) and other furfurals (Ortega-Heras & González-Sanjosé, 2009; Palacios, Valcarcel, Caro, & Perez, 2002), that could be related to the unknown peaks detected. However, the structure of melanoidins is poorly defined and is not isolated and characterised, making it difficult to identify them.

Regarding the commercial wine vinegars under study, especially those samples misclassified as Modified samples which were expected to have a greater amount of grape-must caramel in their composition, the chromatographic results agreed with the fluorescence patterns. Hence, these samples showed higher areas of the two unknown compounds and 5-HMF (i.e. three times more) than the rest of CR and RE

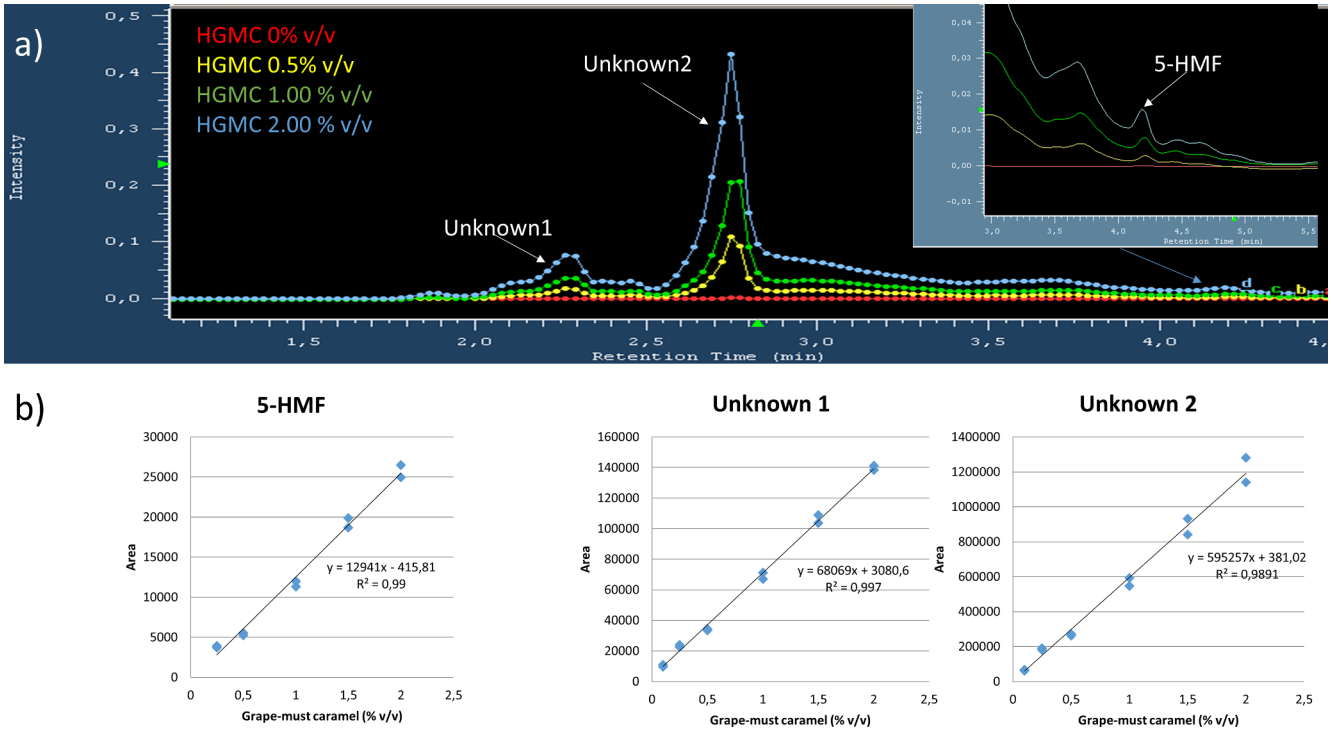

Fig. 3. Chromatograms corresponding to different solutions of grape-must caramel in the hydroacetic matrix showing the elution of the selected peaks (a); linear regression curves of the three compounds selected (5-HMF and two unknowns) obtained by the different percentages of grape-must caramel in hydroacetic matrix (b). HGMC = Hydroacetic matrix with the addition of grape-must caramel.

commercial wine vinegars.

3.5.2. Multiway calibration

In spite of the promising results shown in the previous section, as grape-must caramel is a mixture of compounds and wine vinegar is another complex matrix of compounds, when a univariate calibration was developed with PARAFAC components extracted from the wine vinegar matrix, and not with the hydroacetic matrix, in this case satisfactory results were not achieved. This could be explained by the fact that in order to make correct predictions with the univariate model, the signal of the test samples can only vary due to the analyte, so the contribution of the other species must be the same as what has been modelled. If the contribution of these other species varies (because their concentration varies) or if there is some new interfering signal, the prediction will be biased. The advantage of a multiway calibration over the calibration line is that it allows selective information to be obtained from non-selective instrumental responses (that is, in the presence of interferences), thus enabling the determination of the concentration of various components in complex samples (Olivieri, 2014) to be determined. By using multiway calibration, it has been demonstrated that considerably more complex analytical problems can be solved and predictions are possible – even in the presence of unexpected spectral interferences, i.e., sample constituents not considered in the calibration phase (Arancibia, Damiani, Escandar, Ibañez, & Olivieri, 2012; Bro, 1998; Christensen, Becker, & Frederiksen, 2005; Olivieri & Escandar, 2014; Olivieri, 2014).

For this reason, a multiway calibration method such as N-PLS that considers the entire EEM matrix was studied (Fig. 4). The N-PLS calibration model was built using the EEM data from all of the modified and unmodified wine vinegars in an attempt to identify a possible correlation of the matrices with the quantity of added grape-must caramel. This algorithm has the advantage of being a simultaneous model, that is, all of the components are extracted at the same time. Again, two strategies were developed: building a model with both PDOs together, and analysing each PDO separately. The NPLS accuracy for each model is shown in Fig. 4. As indicated by the high correlation coefficient ($R^2 > 0.921$) and low RMSEC, the results of the three models were good. Moreover, the good regression results obtained by the multiway calibration agree with those obtained by other authors, due to the N-PLS algorithm having been demonstrated to be superior to unfolding methods, primarily owing to a stabilisation of the decomposition that has been demonstrated potentially to give better predictions (Bro, 1996). Moreover, another advantage is that the algorithm is fast compared with the PARAFAC approach because it consists of solving eigenvalue problems.

For regression model robustness, five of the modified samples prepared for each PDO (with intermediate concentrations of 0.20, 0.40, 0.75, 1.25 and 1.75% of grape must caramel) were used as validation sets (included randomly in train and test) in order to test the models using known amounts of grape-must caramel. The overall prediction model accuracy obtained by the three NPLS models was very good with respect to the % of grape-must caramel predicted for these 5 samples, demonstrating the efficacy of the NPLS method. The results obtained, expressed as % of grape-must caramel, with the predicted values in brackets, as follows: 0.2(0.29), 0.4(0.47), 0.75(0.93), 1.25(1.39), and 1.75(1.85) by the global model (being these values an average of the results for both PDOs); 0.2(0.16), 0.4(0.58), 1.25(1.39), 1.75(1.74) for the *Jerez* model; and 0.2(0.18), 0.4(0.50), 0.75(0.99), 1.25(1.43) for the *Montilla-Moriles* model. The prediction results obtained for the test set are shown in Supplementary Table 2. Therefore, regarding the comparison between the measured and the predicted values obtained for these 5 samples, better results were obtained by the global NPLS model (with samples from both PDOs) than by the individual NPLS model of each PDO. This might be explained by the fact that this first model has a higher amount of samples with the same concentrations than the individual models.

In terms of the real wine vinegars, the calibration results for the RE commercial samples of both PDOs that had been shown as possibly containing more grape-must caramel or even as being less aged vinegars, again agreed with the exploratory and classification analyses

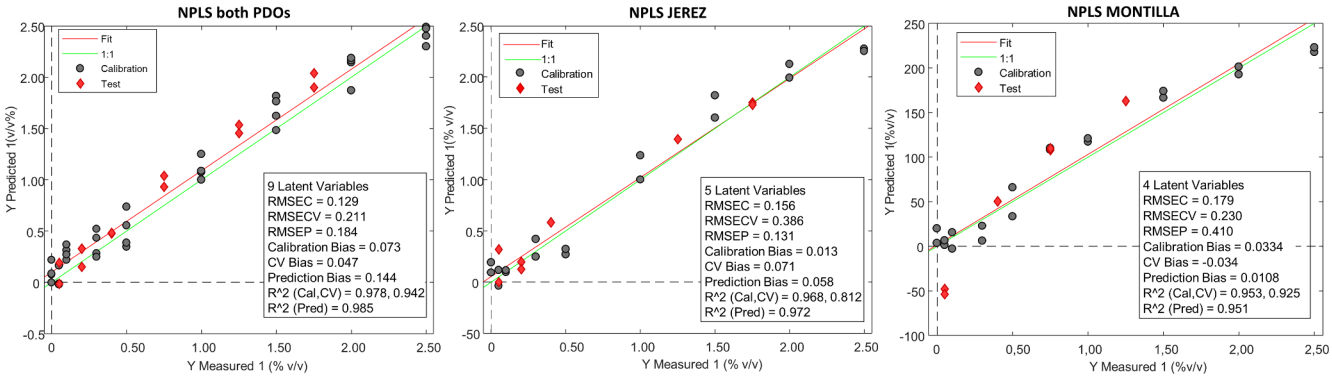

Fig. 4. Figures of merit of the multiway calibration models developed with the grape-must calibration curves of both PDO considered together (a) and for each PDO individually ((b) and (c)).

performed in a previous section of this work. Thus, according to the predicted results (Supplementary Table 2), the RE samples misclassified of *Vinagre de Jerez* PDO presented amounts of grape-must caramel around 2.0%, agreeing with the predicted values of modified samples with the addition of 2.0% of grape-must caramel, whereas the rest of commercial samples had an amount of grape-must caramel lower than 1.5% and even 0.0%. Regarding the RE samples of *Vinagre de Montilla-Moriles* PDO that were classified as Modified, the predicted amount of grape-must caramel was higher than 1.0%, while the rest of the commercial samples presented a predicted value of lower than 0.5%. These values agreed totally with the observed trend of these samples in the previous PCA models. These samples were also those that showed the highest chromatographic areas for the three selected peaks.

Furthermore, in CR commercial samples that also showed a high similarity to the Modified samples with a lower amount of caramel (< 0.05%), the percentages of grape-must caramel obtained by the regression models were even negative, being in agreement with this assumption (Supplementary Table 2).

All of these results confirm the ability of this multiway calibration to determine the amount of grape-must caramel in PDO wine vinegars and its ability to detect samples with an excessively high concentration. An excessive addition of grape-must caramel to a vinegar could affect its quality due to sensory changes. In fact, ranking and triangle tests, both gustatory and olfactory, were undertaken in order to assess the hypothesis of the sensory effect that adding grape-must caramel could have and in order to know the specific level of grape-must caramel that modified the sensory characteristics. Thus, the results obtained by these tests showed that, in general, 0.3% was the minimum level of concentration of grape-must caramel at which all of the tasters perceived sensory differences in the samples. However, in *Vinagre de Jerez*, grape-must caramel at a concentration of 0.05% was also perceived by many testers as being different to the raw matrix. These results reaffirm the relevance of the present study on the importance of quantifying the grape-must caramel added to wine vinegars, due to the fact that changes in the organoleptic characteristics of wine vinegars were detected very low concentrations.

4. Conclusions

Multidimensional fluorescence coupled with a suitable chemometric method has shown itself to be a valuable tool for detecting and, for the first time, quantifying the addition of grape-must caramel to wine vinegars without sample treatment. Thus, the methodology proposed provided results that were in agreement with those obtained by the conventional HPLC analytical method. This, therefore, demonstrated the validity of the procedure for determining the amount of grape-must caramel in wine vinegars.

This study has also shown that the multiway regression and classification approaches using NPLS and NPLS-DA, respectively, provide even better results more easily and more quickly than the common procedure of EEM matrices by developing PARAFAC models before the classification and regression models. PARAFAC has the advantage of providing more information about the fluorescent compounds presented in the matrices, yet it involves a more complex chemometric approach.

The addition of grape-must caramel is a common practice in the vinegar industry. It has not been studied previously because it was thought that it had no influence on the final vinegars. However, sensory changes in vinegars caused by adding grape-must caramel were also studied. The results show that low concentrations produce changes in the organoleptic characteristics of PDO wine vinegars, reaffirming the relevance of determining the addition of grape-must caramel.

This study opens up a new means of detecting and monitoring the addition of grape-must caramel to wine vinegar, thus preventing unfair competition between wineries and brands, as well as preventing potential adulterations related to the addition of grape-must caramel. Therefore, now that the important effects that adding grape-must caramel has upon a PDO vinegar's final quality have been demonstrated, further studies are needed in order to gain greater knowledge of the subject with the aim of establishing a limit or creating a monitoring protocol regarding the addition of grape-must caramel to PDO vinegars.

Declaration of interests

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2019.02.008.

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SUPPLEMENTARY TABLE 1. Sensitivity and specificity (%) obtained for PLS-DA and NPLS-DA classification models.

PDO	Set	Performance classification parameter	Modified		Unmodified	
			P	N	P	N
'Vinagre de Jerez'	TRAINING	Sensitivity	100	100	100	100
		Specificity	100	100	100	100
	PREDICTION	Sensitivity	100	100	43	63
		Specificity	43	63	100	100
'Vinagre de Montilla-Moriles'	TRAINING	Sensitivity	100	100	100	100
		Specificity	100	100	100	100
	PREDICTION	Sensitivity	100	100	25	40
		Specificity	25	40	100	100
Both PDOs together	TRAINING	Sensitivity	91	90	86	91
		Specificity	86	91	91	90
	PREDICTION	Sensitivity	100	100	36	58
		Specificity	36	58	100	100

P: PLS-DA: N: NPLS-DA

SUPPLEMENTARY TABLE 2. Prediction on test samples by NPLS calibration analysis.

CLASS	SAMPLE	REAL (% v/v)	PRED (% v/v) GLOBAL MODEL	PRED (% v/v) JEREZ MODEL	PRED (% v/v) MONTILLA MODEL
Modified control matrix	JCCR_0.2	0.2	0.24	0.16	
	JCCR_1.25	1.25	1.45	1.39	
	JCCR_1.75	1.75	1.90	1.76	
	JCRE_0.2	0.2	0.33	0.20	
	JCRE_0.4	0.4	0.48	0.58	
	JCRE_1.75	1.75	1.80	1.74	
	MCCR_0.2	0.2	0.3		0.16
	MCCR_0.4	0.4	0.47		0.50
	MCCR_0.75	0.75	1.04		1.08
	MCRE_0.75	0.75	0.93		0.90
	MCRE_1.25	1.25	1.34		1.43
Modified commercial matrix	JCR_0.5	0.5	0.47	0.02	
	JCR_1	1	0.83	0.83	
	JCR_1.5	1.5	1.38	1.55	
	JCR_2.0	2	2.19	2.13	
	JCR_2.5	2.5	2.26	2.24	
	MCR_0.5	0.5	0.67		0.37
	MCR_1.0	1	1.12		1.11
	MCR_2.0	2	2.37		2.21
Unmodified commercial samples	JCR	x	0.50	-0.47	
	JCR	x	0.93	0.56	
	JCR	x	0.47	0.43	
	JCR	x	1.39	1.12	
	JCR	x	**1.86**	**1.71**	
	JCR	x	0.95	0.93	
	JCR	x	1.28	1.25	
	JCR	x	1.20	1.18	
	JCR	x	0.61	0.34	
	JCR	x	0.69	-0.53	
	JRE	x	1.27	1.32	
	JRE	x	1.82	1.25	
	JRE	x	**2.61**	**2.20**	
	JRE	x	0.81	-0.70	
	JRE	x	1.41	0.38	
	JRE	x	0.73	0.81	
	JRE	x	1.53	1.56	
	JRE	x	0.67	-0.60	
	JRE	x	0.30	0.38	

	JRE	**x**	1.51	1.07	
	JRE	**x**	**1.94**	**1.91**	
	JRE	**x**	0.68	-0.84	
	JRE	**x**	0.45	0.74	
	MCR	**x**	-1.05		-2.79
	MCR	**x**	0.24		0.54
	MCR	**x**	**-0.52**		**-3.28**
	MCR	**x**	-0.55		-0.90
	MCR	**x**	0.50		0.24
	MCR	**x**	0.50		0.12
	MRE	**x**	0.95		-0.43
	MRE	**x**	0.90		-0.79
	MRE	**x**	0.93		0.67
	MRE	**x**	**1.74**		**1.74**
	MRE	**x**	**1.37**		**1.58**

*Note: Marked in bold the samples with special results that were misclassified in the models.

Vinagre de Montilla-Moriles PDO

Vinagre de Jerez PDO

Modified Samples

Control samples

1) Addition of grape-must caramel to a "Crianza" vinegar without caramel

CONTROL CR **0.05** (-0.1-0.2-0.3-0.4-) **0.50** (-0.75-) **1.00** (-1.25-) **1.50** (-1.75-) **2.00** **2.50** **%v/v** **CONTROL RE**

2) Addition of grape-must caramel to a "Reserva" vinegar sample without caramel

CONTROL RE 0.05 - 0.1 - 0.2 - 0.3 - 0.4 - 0.50 - 0.75 - 1.00 - 1.25 - 1.50 - 1.75 - 2.00 - 2.50 %

Commercial samples

3) Addition of grape-must caramel to a "Crianza" commercialized PDO vinegar

Commercial CR **0.05** **0.50** **1.00** **1.50** **2.00** **2.50** **%v/v** **Commercial RE**

Unmodified samples

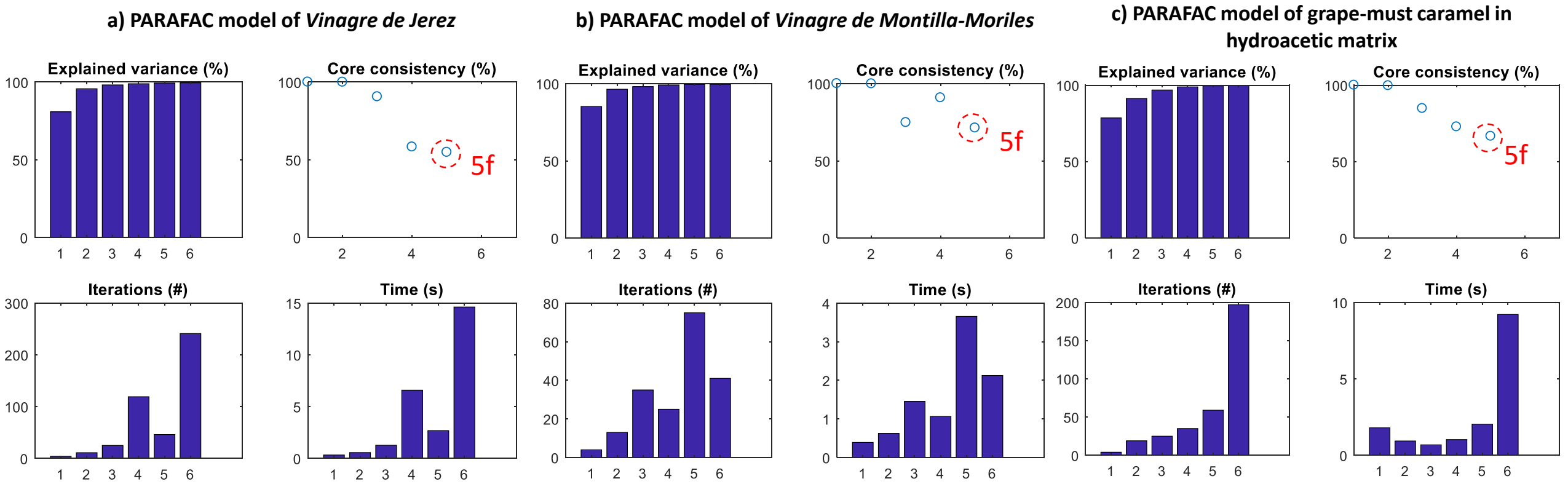
5) "Crianza" and "Reserva" commerical PDO wine vinegars

Hydroacetic curve

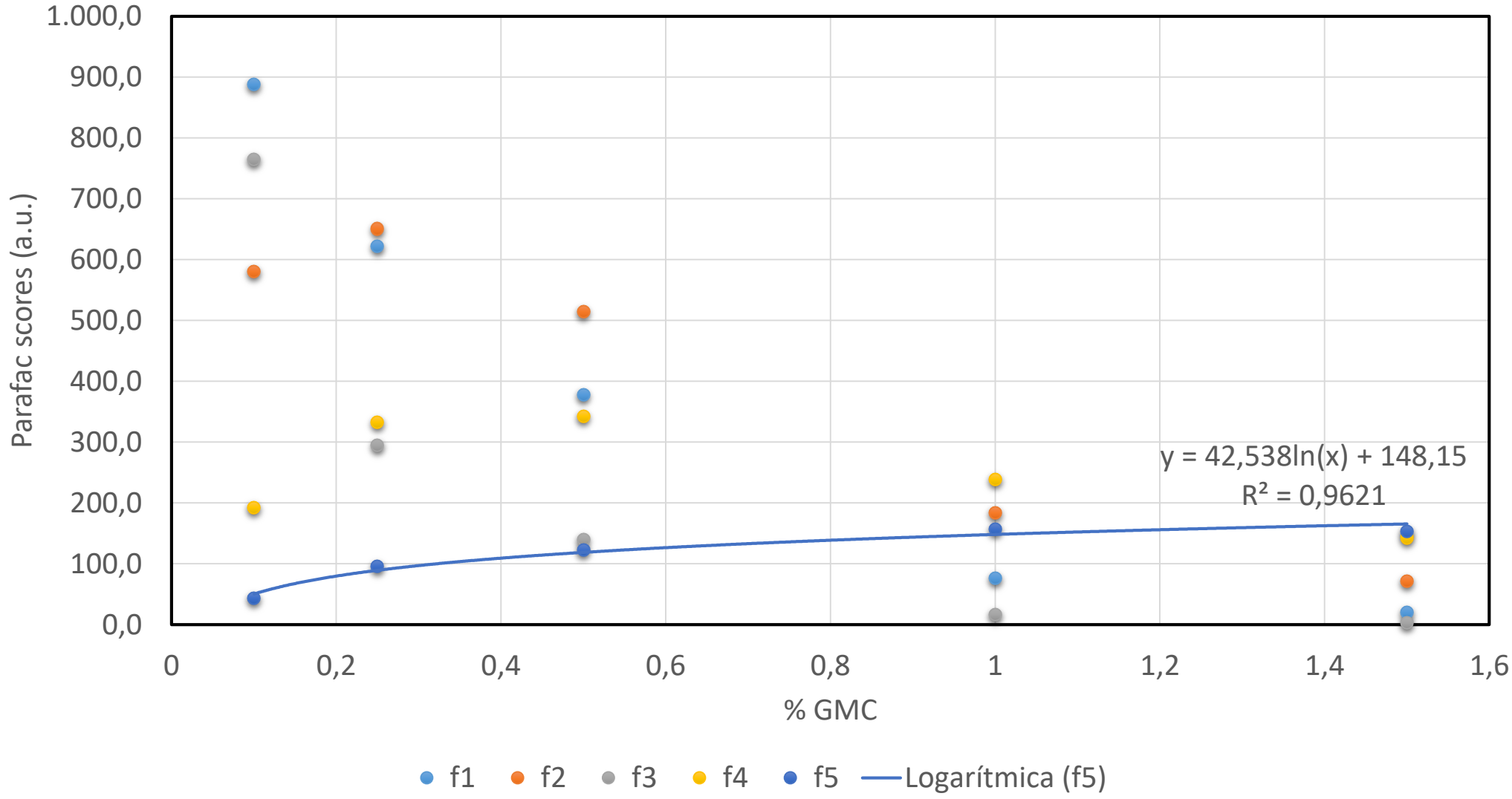
4) Addition of grape-must caramel to hydroacetic matrix

0.10 - 0.25 - 0.50 - 1.00 - 1.50 - 2.00 % v/v

Supplementary Fig. 1. Schematic representation of the different samples and grape-must caramel curves included in the study.



Supplementary Fig. 2. Plot of the variance explained (%), core consistency (%), number of iterations and time to carried out each model, by extracting from 1 to 6 factors, used in the selection of the best number of factors for the *Vinagre de Jerez (a)* and *Vinagre de Montilla-Moriles (b)* PARAFAC models (including Modified and Unmodified samples), and for the PARAFAC model made with the grape-must caramel calibration curve in hydroacetic matrix (c).



Supplementary Fig. 3. Evolution of the scores of PARAFAC factors extracted from the hydroacetic curve with the addition of grape-must caramel.

BLOQUE I:

CARACTERIZACIÓN Y CLASIFICACIÓN ESPECTROSCÓPICA DE LOS VINAGRES DE VINO ESPAÑOLES CON DOP

CAPÍTULO III:

Fusión de datos espectroscópicos (FTIR, NIR, EFM, RMN)

CHAPTER III.

Data fusion of spectroscopic data (FTIR, NIR, EFM, NMR)

RESUMEN

Los vinagres de vino con DOP son matrices muy complejas multi-componente de compuestos químicos, ya sea por su procedimiento tradicional de fabricación, la materia prima utilizada o el envejecimiento en barricas de madera. Por ello, diferentes técnicas analíticas se han estudiado en los capítulos anteriores de esta memoria de tesis con el fin de obtener una caracterización exhaustiva de estos vinagres y evaluar la capacidad de clasificación y discriminación de cada una de ellas, con objeto de seleccionar aquella que proporcione los mejores resultados.

Tal y como se ha presentado en los trabajos anteriores (MIR, NIR, EFM), estas técnicas analíticas ya proporcionaron buenos resultados en términos de clasificación según categorías o dentro de cada DOP por separado. Sin embargo, aún no había sido posible clasificar perfectamente las tres DOP de vinagres de vino, independientemente de su categoría, envejecimiento o la dulzura, es decir, ser capaz de clasificar un vinagre sea de la categoría que sea, dentro de una DOP. Por esa razón, y con el objetivo de mejorar los modelos de clasificación obtenidos, se propuso un nuevo trabajo en el que se combinaran y fusionaran los datos de las técnicas espectroscópicas empleadas hasta el momento, con objeto de ver si se podrían mejorar los modelos de clasificación obtenidos de forma individual entre las DOPs y categorías. Además, la integración de los diferentes tipos de datos en un único modelo también permitiría evaluar la correlación y el contenido de información similar o diferente entre las distintas técnicas.

En este contexto, el objetivo de este trabajo, publicado en Talanta 198 (2019) 560–572, fue realizar una caracterización multiplataforma y desarrollar modelos de autenticación/clasificación para las diferentes DOP de vinagre de vino españolas, así como estudiar la sinergia/complementariedad entre las técnicas consideradas para tal fin. Para ello, en este trabajo se evaluaron diferentes estrategias de fusión de datos (FD) con el fin de alcanzar la mejor discriminación de los tres vinagres de DOP.

En este trabajo, 65 vinagres de vino de las tres DOP españolas fueron analizadas por cuatro técnicas espectroscópicas: espectroscopía infrarroja de transformada de Fourier (FTIR), espectroscopia de infrarrojo cercano (NIR), espectroscopía de fluorescencia multidimensional (EEM) y resonancia magnética nuclear protónica (^{1}H-NMR). El muestreo y los procedimientos analíticos utilizados para el análisis de las muestras por FTIR, NIR y EFM fueron los mismos utilizados en los trabajos anteriores, donde se describen en detalle. Pero, además, en este trabajo se añadió el análisis por ^{1}H-RMN, debido a que es una técnica rápida, sin apenas manipulación de la muestra, como ocurre con las técnicas espectroscópicas anteriores, y

además los resultados que se obtienen son robustos y contienen una gran información. Una vez obtenidos todos los datos, se realizaron distintos análisis de componentes principales (PCA) y modelos de clasificación mediante mínimos cuadrados parciales (LDA-PLS-DA) sobre los datos de técnicas individuales, previamente descompuestos por diferentes métodos. Posteriormente, todas las señales instrumentales se procesaron a la vez utilizando diferentes estrategias de fusión de datos: fusión de datos de nivel medio mediante dos estrategias de pre-procesamiento y el método multibloque *P-Comdim.*

Con respecto a la fusión de datos (FD), se necesitaron diferentes algoritmos quimiométricos para extraer y fusionar la información presentada en cada conjunto de datos debido a la distinta naturaleza y estructura de los datos, como por ejemplo PCA, PARAFAC o MCR. Con todas las características extraídas de cada bloque de datos, se realizó la estrategia de FD de nivel medio, evaluándose además dos pre-procesamientos distintos: autoescalado y autoescalado de bloques.

Los resultados obtenidos de los modelos de FD con ambos pre-procesamientos mostraron una mejora en la clasificación de las muestras según la DOP, proporcionando una diferenciación más eficiente que los modelos basados en los análisis espectroscópicos individuales. Con respecto a los métodos analíticos individuales, especialmente los resultados de clasificación de los modelos ^{1}H-RMN fueron prometedores, obteniéndose unos porcentajes de correcta clasificación de entre el 75 y 100%.

Por otro lado, la aplicación del método P-ComDim se utilizó para describir, de manera simple y sintética, la información espectral global recogida y revelar la complementariedad y diferencias de las técnicas espectroscópicas, evaluando la importancia de cada técnica para cada uno de los variables comunes, cosa que no se obtiene por la FD de nivel medio. Sin embargo, los resultados del presente trabajo mostraron que la FD de nivel medio resultó la mejor opción para la clasificación de DOPs de vinagres de vino, independientemente de la categoría a la que pertenezcan, en comparación con los modelos de clasificación obtenidos por P-ComDim, y con los modelos obtenidos individualmente de cada técnica.

Estos resultados muestran que combinar distintas técnicas espectroscópicas por fusión de datos permite obtener sinergias/complementariedad de ellas, logrando una mejor diferenciación de las DOP españolas de vinagres de vino.

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ARTÍCULO 5

Data fusion approaches in spectroscopic characterization and classification of PDO wine vinegars

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Data fusion approaches in spectroscopic characterization and classification of PDO wine vinegars

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ABSTRACT

Spain is one of the major producers of high-quality wine vinegars having three protected designations of origin (a.k.a. PDOs): "Vinagre de Jerez", "Vinagre de Condado de Huelva" and "Vinagre de Montilla-Moriles". Their high prices due to their high quality and their high production costs explain the need for developing an adequate quality control technique and the interest in extensive characterization in order to capture the identity of each denomination. In this framework, methodologies based on non-targeted techniques, such as spectroscopies, are becoming popular in food authentication. Thus, for improving vinegar quality assessment, fusion of data blocks obtained from the same samples but different analytical techniques could be a good strategy, since the quantity and quality of sample knowledge could be enhanced providing new insights into the differentiation of vinegars. Therefore, the aim of this manuscript is the development of a multi-platform methodology and a model able to classify the Spanish wine vinegar PDOs. Sixty-five PDO wine vinegars were analyzed by four spectroscopic techniques: Fourier-transform mid-infrared spectroscopy (MIR), near infrared spectroscopy (NIR), multi-dimensional fluorescence spectroscopy (EEM) and proton nuclear magnetic resonance (^{1}H-NMR). Two different data fusion strategies were evaluated: Mid-level data fusion with different preprocessing, and Common Component and Specific Weights analysis multiblock method. Exploratory and classification analysis on the data from individual techniques were also performed and compared with data fusion models. The data fusion models improved the classification, providing a more efficient differentiation, than the models based on single methods, and supporting the approach to combine these methods to achieve synergies for an optimized PDO differentiation.

1. Introduction

Nowadays, there is a growing consumer's demand for high quality food products. The term "quality" in food is directly related to a known origin and specific chemical composition, adequate and satisfactory physical and sensory properties, as well as meeting safety and health requirements [1,2]. Protected Designation of Origin (PDO) indication is one of the label adopted by the European Community as recognition of some specific food quality attributes. A product with a PDO registration must be produced, processed and prepared in a given geographical area using a recognized know-how [3]. The PDO denomination confers to these products a high added value, consequently there is also an increasing of deceptive practices aiming at counterfeiting them, such as mislabeling of geographical origin, disregarding the production protocol or adulteration of the product. In this respect, assessing the authenticity of traditional food is a complex issue because it has to encompass several aspects going from assessing the compliance to the legal requirements stated in the product label, i.e. controlling the geographical origin and the respect of the traditional protocols, to detecting fraudulent processing practices or adulteration.

Among the PDO products with high demand there are the high-quality vinegars. In particular, in addition to the well-known "Aceto Balsamico Tradizionale di Modena" from Italy [4], Spain is also one of the major producers of high-quality wine vinegars. Thus, three important Spanish wine vinegars have gained the PDO label because of their unique characteristics and traditional production, namely: "Vinagre de Jerez" (also known as "Sherry wine vinegar"), "Vinagre de Condado de Huelva" and the most recently "Vinagre de Montilla-Moriles". Furthermore, within each PDO, there are different categories according to their time and method of aging ("criaderas and solera" or

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"añada" systems) in wood barrels as well as the sweetness. The high quality of these wine vinegars is linked to the raw material used (i.e. high quality wines, also protected by the corresponding PDO), the traditional production protocol and method of aging in wooden barrels. Therefore, the high prices of these vinegars, due to their high quality, the long aging time and hence, the high cost of their production, explain the need of proper characterization in order to provide an adequate quality control to defend their identity [4–9].

Due to the traditional making procedure, the raw material used and the aging process, these wine vinegars are very complex multi-component mixtures from the chemical point of view, thus different analytical techniques have been applied to obtain an extensive characterization in order to assess their authenticity [6,7,9–11]. Spectroscopic techniques, based on infrared (IR), fluorescence or nuclear magnetic resonance (NMR) spectroscopy, are the most commonly used food fingerprinting techniques in untargeted approach. In particular, these spectroscopic techniques share the advantage of requiring minimal sample preparation, moreover IR is non-destructive and chip, while NMR may allow quantification of a wide range of compounds. Good results were obtained by spectroscopic analysis of the three Spanish PDO wine vinegars in terms of assessing their aging and sweet categories or characterizing each PDO separately [6,7,12]. However, the possibility of discriminating these three wine vinegars PDOs, regardless of the presence of different ageing or sweetness features, within each distinct PDO, has been less considered in the literature [7,9,11].

In order to gather more detailed knowledge about the specificity of each PDOs and aiming at improving their quality assessment and differentiation, the combination and fusion of the data acquired by several analytical platforms could be useful [2,13,14]. Data fusion methodologies have demonstrated to be a powerful tools for obtaining more reliable authentication models with respect to the results obtained by each technique separately [2,13,15–17]. In fact, the fusion of the different information obtained can enhance the quantity and quality of knowledge about the distinctive features among samples/categories. Moreover, the integration of the different data types into a single model also allows assessing the correlation and the similar/different information content among the different techniques.

Data fusion may be accomplished at different levels (i.e. low-, mid- and high-level data fusion), depending on the objective, number and type of data sets to combine [2,18–20]. The low-level fusion is a conceptually simple method: raw data from more than one source are directly fused (concatenated) after preprocessing issues are addressed. This level of data fusion has been widely applied for the authentication and quality control of many food and beverages [2]. The main limitations are a high data volume and the possible predominance of one data source over the others and possible discontinuities regions when spectral data are fused. This is partially overcome by the mid-level fusion, in which a previous extraction of some relevant features from each single data source is performed and then, these features are concatenated into a single array. Moreover, this type of fusion enables an easy interpretation of the results, since the contribution of each individual block can be visualized. The main parameters to take into account are the number of features to retain from each model, the method to be used for data reduction, and the type of scaling to apply, however this last issue is less severe than in low-level data fusion, considering that data reduction has already been applied. Mid-level data fusion has been also applied in authentication and quality control of food and beverages [2,17].

On the other hand, other approaches based on multiblock analysis are also suitable in data fusion context, such as the Common Components and Specific Weights Analysis (CCSWA, also referred to as ComDim, which is as well the name of the algorithmic implementation) [21–23], which has been recently revised and extended to the supervised context (P-ComDim) [24], *i.e.* to deal with the case where one of the blocks (Y block) holds responses that are to be predicted on the basis of the information provided by the other blocks. The main purpose of the ComDim algorithm is to provide the common sources of information shared by each data block, i.e. the common components, at the same time assigning to each single block a specific weight (or salience) associated to each dimension of the common space [24,25]. This method has been recently applied to the analysis of several food products in order to differentiate e.g. an organically or conventional production [26,27], or cheese products obtained by different manufacturing or ripening [28] as well as it has been applied to predict sensory attributes [21].

A major general advantage of ComDim approach, compared to the low and mid-level data fusion approaches, is that it provides information about the relation between individual data blocks (i.e. common variables) and their contribution to each common component. Thus, ComDim can be applied in order to study the complementarity, and also the differences, of the various spectroscopic techniques. In particular, the study of the saliences (weights of each data block in the common model) could be particularly interesting due to the fact that if a dimension has close saliences for two or more techniques, this may be due to a physical phenomenon that is described in a similar way for both methods. On the other hand, if there is an important difference between the saliences for a given dimension, it could mean that this dimension reveals a phenomenon only visible by one technique and not by the others. This could be used for focusing the selectivity of the spectroscopic techniques studied in this work.

Moreover, used in the predictive context, i.e. P-ComDim, we could infer and assess which information in the different data blocks is relevant for the discrimination of the different categories, which is shared and which is peculiar to each of them [24,28,29]

Taking this background into consideration, the aim of this work was to perform a multiplatform characterization and develop classification models for the different Spanish wine vinegar PDOs by assessing different data fusion approaches, as well as to study the synergy/complementarity among the techniques considered for that purpose. To this aim, the same wine vinegar samples were measured by four spectroscopic techniques: Fourier-transform infrared spectroscopy (i.e. mid infrared, MIR), near infrared spectroscopy (NIR), multidimensional fluorescence spectroscopy (EEM) and proton nuclear magnetic resonance (^{1}H-NMR). These techniques were selected due to the individual efficacy in the characterization of PDO wine vinegars as previously reported [6,7,12] , as well as because they have gained wide acceptance in foods characterization, authenticity and classification purposes [15,30–34].

The main contribution of this study is to comparatively discuss the different data fusion strategies, in term of capability to improve discrimination of the three PDO's vinegars and to highlight the role of each spectroscopic technique. In fact, although they can share some repeated pieces of information, they are mostly complementary.

2. Materials and methods

2.1. Samples

Sixty-five PDO wine vinegar samples were provided by several local wineries through the Council Regulation of each PDO. Twenty-one samples belonging to the PDO "Vinagre de Condado de Huelva", twenty-eight to "Vinagre de Jerez" PDO and sixteen to the most recently designed PDO "Vinagre de Montilla-Moriles" were analyzed by the four analytical techniques which are described below. Furthermore, within each PDO, samples from the different commercialized categories (aged and sweet) were included in the analysis. Samples were analyzed in duplicate. More information about the samples is presented in Table 1.

Table 1
Samples included in the study.

PDO	Category	Ageing	n
"Vinagre de Jerez" (J)	Crianza	≥ 6 months	11
	Reserva	≥ 2 years	13
	Pedro Ximenez	–	4
	Total	28	
"Vinagre de Condado de Huelva" (C)	Without ageing	0 months	5
	Solera	≥ 6 months	5
	Reserva	≥ 2 years	8
	Añada	≥ 3 years (static system)	3
	Total	21	
"Vinagre de Montilla-Moriles" (M)	Crianza	≥ 6 months	8
	Reserva	≥ 2 years	3
	Pedro Ximenez	–	5
	Total	16	

2.2. Instrumental analysis

2.2.1. Mid-infrared spectroscopy (MIR)

Samples were analyzed, according to the method reported in [6], by using a Bruker Vertex 70 FTIR spectrometer equipped with a DGTS detector (Bruker Optics, Ettlingen, Germany) and a multi-reflection attenuated total reflectance accessory (ATR, six bounces, Specac, Orpington, U.K.). Samples were directly analyzed without sample pre-treatment, recording the spectra at the same temperature (22 ± 0.05 °C) in the region of 4000–600 cm^{-1} (by an average of 50 scans at a resolution of 4 cm^{-1}) and were examined using OPUS version 7.0 (Bruker Optics, Ettlingen, Germany) and manipulated with OMNIC software. The raw MIR spectra are shown in Fig. I. Supplementary Material.

2.2.2. Near-infrared spectroscopy (NIR)

NIR spectra were collected following the method published in [12], by using an ABB Bomen IR spectrometer (Q-interline, X, Denmark), equipped with a 1 mm path length cuvette. Spectral data were collected in the range of 12000–4000 cm^{-1}, resolution of 8 cm^{-1}, and 64 scans for both backgrounds and samples. Samples were directly analyzed without sample pre-treatment in a random sequence at room temperature (21 ± 2 °C) by pipetting them into 1 mL shell vial, 40 × 80 mm transparent (Skandinaviska Genetec AB, Lund, Sweden) before measurement. The spectrometer was interfaced to a computer with GRAMS/AI™ Spectroscopy Software (Thermo Fisher Scientific software) for spectral acquisition and exportation. The raw NIR spectra are shown in Fig. I. Supplementary Material.

2.2.3. Excitation-Emission Multidimensional Fluorescence (EFM)

Wine vinegar samples were directly analyzed without sample pre-treatment an at the same temperature (25.00 ± 0.05 °C) by a Varian Cary-Eclipse fluorescence spectrophotometer (Varian Iberica, Madrid, Spain), equipped with two Czerny-Turner monochromators, and a Xenon discharge lamp pulsed at 80 Hz with a half peak height of ≈ 2 μs, according to the method reported in [7]. Cary-Eclipse software was used for spectral acquisition and exportation. The fluorescence Excitation-Emission Matrices (EEMs) were obtained by varying the excitation wavelength (λex) between 250 and 700 nm (every 5 nm) and recording the emission spectra (λem) from 300 to 800 nm (every 2 nm), with excitation and emission slits set at 5 nm and the scan rate fixed to 1200 nm min^{-1}. EEMs were preprocessed in order to avoid noisy and non-informative areas by selecting shorter spectral ranges (λex from 250 to 680 nm, and λem from 310 to 800 nm). The EEM landscape of a vinegar is shown in Fig. I. Supplementary Material as an example.

2.2.4. ^{1}H-Nuclear Magnetic Resonance (^{1}H-NMR)

Samples were prepared by adding 100 μL of 0.16% of 3-(Trimethylsilyl) propionic-2,2,3,3-d4 acid sodium salt (TMSP-2,2,3,3-d4) in D2O (99.97%) dissolution, to 600 μL of each wine vinegar. TMSP was used as both a chemical shift reference ($\delta = 0$) and internal standard. ^{1}H-NMR spectra have been acquired at 300 K of temperature on a Bruker AVIII 700 spectrometer (Bruker Biospin GmbH Rheinstetten, Karlsruhe, Germany) operating at 700.25 MHz. The ^{1}H-NMR data were acquired using the Bruker spin−echo sequence "cpmgpr.fb" (Carr–Purcell–Meiboom–Gill, Bruker Library) with water presaturation, applied to suppress broad resonance signals. FIDs' have been recorded as the sum of 64 scans of 7.4 s each covering a spectral width of 11.0 ppm with 1 s between each consecutive scan. Data acquisition was carried out using the "baseopt" Bruker sequence to optimize the baseline after Fourier Transform. The raw ^{1}H-NMR spectra are shown in Fig. I. Supplementary Material.

2.3. Data analysis

Since four different instrumental fingerprints were recorded for each sample, each one with different data structures, several chemometric algorithms were employed in order to extract and merge the information presents in each data set.

The data analysis workflow included: i) building separate models: both exploratory analysis and classification were performed on the data obtained from the individual analytical techniques; ii) in order to take advantage of the multiplatform characterization of the samples, the data of different sources were processed by means of different data fusion (DF) strategies. The objectives were to assess common and specific information pertaining to each analytical platform and obtaining improved classification results. A schematization of the global data analysis flow is presented in Fig. 1.

2.3.1. Data sets

In total sixty-five samples were analyzed by each spectroscopic technique. In order to validate the models, the samples were split in a training set of forty-seven samples (fifteen "Vinagre de Condado de Huelva", twenty "Vinagre de Jerez" and twelve "Vinagre de Montilla-Moriles" PDO samples) and a test set of eighteen samples (six "Vinagre de Condado de Huelva", eight "Vinagre de Jerez" and four "Vinagre de Montilla-Moriles" PDO samples) using the Duplex algorithm [35]. This algorithm ensures a representative spanning of the whole data domain for both calibration and validation sets, we also checked for a balanced representation of each category in both sets. Moreover, since the number of samples is rather limited, the splitting was repeated five times (always checking by exploratory data analysis, that both sets spanned the whole variability domain and balanced category representation was achieved) hence five classification models were calculated for each analyzed data set (NIR + MIR, NMR, EEM, mid-level Data Fused, P-Comdim raw data and P-Comdim extracted features). In the results the average classification errors are reported.

2.3.2. Decomposition methods

As summarized in Fig. 1, different decomposition methods were applied, according to the type of dataset, for exploratory data analysis as well as for data reduction to obtain the features which were then used for the data fusion models, *i.e.* mid-level DF and features-based P-ComDim.

MIR and NIR individual data sets, as detailed in Section 2.3.5.1, were concatenated at low-level DF and the obtained dataset was compressed by principal component analysis (PCA).

The EEM data array, after Rayleigh and Raman scattering correction [7], was decomposed by PARAllel FACtor analysis (PARAFAC) [36,37] in order to extract the relevant features (fluorophores).

Finally, for ^{1}H-NMR dataset, after proper alignment and baseline correction, multivariate curve resolution (MCR) [38,39] was used to resolve the chemical components. The peak areas of the resolved components were then used as features.

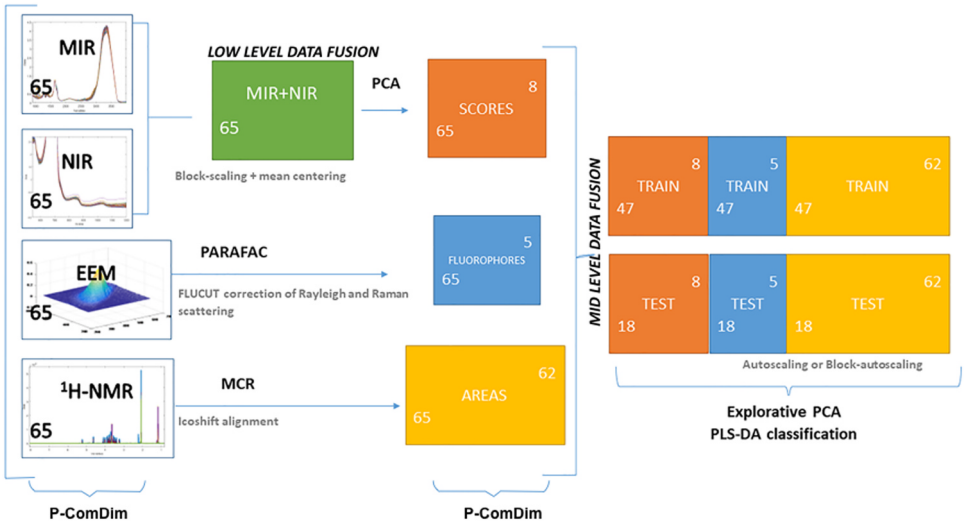

Fig. 1. Graphical representation of the data sets, data analysis flow and data fusion process.

PARAFAC and MCR decomposition methods have been widely described in the literature. Applied constrains and preprocessing details for each data block are reported in Section 2.3.4.

2.3.3. Classification analysis

Partial least squares-discriminant analysis (PLS-DA) is a classification technique based on partial least squares (PLS) algorithm with a so-called dummy matrix reporting class membership as Y block [40]. In our study, three different Spanish PDO were considered, therefore, the size of the Y dummy matrix was *n° samples* × 3 (one column for each one of the classes) and codification was 1/0 (belonging/not belonging to the category).

In the case of EEM data set, which is a three-way array, N-way Partial least squares-discriminant analysis (NPLS-DA) [41] based on multilinear PLS (NPLS) [42] has been used and codification of the Y block is the same as for PLS-DA.

In both cases, classification was achieved by applying linear discriminant analysis (LDA) on the X-scores calculated by PLS-DA/NPLS-DA [43]. The minimum classification error rate in cross-validation (venetian blind, seven splits) was used to assess the number of latent variables, i.e. components of the PLS-DA/NPLS-DA models.

2.3.4. Preprocessing and analysis of individual data blocks

2.3.4.1. MIR and NIR datasets. Concerning the MIR data, as is described in our previous work [6], no preprocessing was needed and the raw spectra were just mean centered. Moreover, only the region between 1500 and 900 cm^{-1} was included in the analysis [6] in order to discard the uninformative variables with excessive noise.

With regards to NIR data, different preprocessing methods were evaluated prior to data analysis as was contemplated in a previous work [12] The best pre-processing approach resulted to be smoothing (Savitzky-Golay filter, 7 points window and second order polynomial degree) to reduce random noise, followed by standard normal variate (SNV) [44] to correct additive scattering. In addition, the spectra were always mean centered prior to any analysis. As mentioned before, based on previous expertise or literature [12,45,46], two segments of the spectrum were removed from the whole acquired wavenumber range: the first one (4000–5430 cm^{-1}) because of low signal/noise ratio and the second one due to the strong combination band of O-H from water (7200–6400 cm^{-1}).

2.3.4.2. EEM dataset. EEM data were preprocessed in order to avoid noisy and non-informative areas by selecting shorter spectral ranges, according to the preprocessing steps described in [7]. Thus, the emission over 680 nm and the excitation below 310 nm were cut. Then, EEM data were corrected for Rayleigh and Raman scattering [47], removing and replacing the scattering areas with interpolated values [47]. After this correction, EEM data was decomposed by PARAFAC [37]. A model based on five factors, constrained for non-negativity in all modes (both concentration and spectral profiles), was built. The proper number of factors was determined by taking into account the CORe CONsistency DIAgnostic test (COR- CONDIA) [48], the explained variance and the visual inspection of the recovered spectral profiles and residuals. The PARAFAC scores (first mode loadings) for these factors were used as features to build the mid-level fused dataset.

2.3.4.3. ^{1}H-NMR dataset. Prior to data analysis, several preprocessing steps were applied to NMR spectra. The regions below 0.84 ppm and over 9.8 ppm were discarded because they were uninformative. Also the region between 4.75 and 5 ppm was removed since it contained the residual water signal not completely removed by the instrumental presaturation step. To correct for the inhomogeneous pH-dependent chemical shifts, all spectra were aligned by means of icoshift [49] whereas weighted least squares (WLS) [50] was used for baseline correction.

Then, MCR was applied. The whole ^{1}H-NMR data was divided into 52 intervals of different size in order to avoid splitting the single NMR signals. This task was performed manually by making use of the previous knowledge of NMR chemical shifts of the main wine and vinegar compounds [33,51,52]. These intervals are shown in Fig. II. Supplementary Material. The MCR settings were the same for each interval: the number of components was determined by inspection of PCA explained variance and SIMPLISMA [53] was used to obtain the initial estimation of the pure spectral profile. The peak areas of the resolved concentration profiles (chemical components) within each interval were calculated by integration and used as features for the subsequent fused data set.

In order to achieve a tentative assignment of the ^{1}H-NMR resolved components, both Chenomx NMR Suite 7.0 (Chenomx, Edmonton, Canada), as well as assignments reported in literature [33,51,52,54,55] were used. Sixty-two components were resolved and integrated; thirty-five of these were tentatively assigned. Those components that were not possible to assign, are named as “X” plus a number. The fact that several regions of the NMR spectra could not be associated to a single signal is due to the many overlapped multiplets present, which impair certain identification. On the other hand, they could be attributed to overall contribution of a class of compounds, such as sugars (between 3 and

4 ppm). In our case, in this region, only glucose and fructose could be separately assessed.

2.3.5. Data fusion strategies

2.3.5.1. Low-level fusion of MIR and NIR data. In the low-level strategy, fusion occurs by concatenating the original data matrices, opportunely pretreated and then analyzing the resulting array as a single data block.

The MIR and NIR spectra were single preprocessed as described in Section 2.3.4.1. Then, the matrices describing the individual blocks were concatenated to obtain a single one, having as many rows as samples analyzed and as many columns as spectral wavelengths selected for each data set. This new matrix was additionally normalized in order to compensate for the different measuring scales and variability of each technique in order to prevent one block from being dominant in the subsequent data analysis [2]. Thus, block-scaling, to equalize variance, and mean centering were applied. Doing so, each block presented variance equal to one, but the ratio of the variance between any two variables inside a single block was preserved.

After preprocessing, a PCA model based on 8 principal components, accordingly to Scree plot and explaining 99.87% of total variance, was selected and the extracted score vectors were used as MIR/NIR features to build the fused dataset.

A possible alternative approach consists of applying PCA to the separate MIR and NIR spectral data and then using the extracted features (distinct set of PCs) in mid-level DF; this approach was also considered and gave very similar results.

2.3.5.2. Mid-level data fusion. In the mid-level strategy, fusion occurs at the level of features extracted from the different data blocks. In this study, as Fig. 1 shows, the final fused array was assembled using the 8 PCA scores from MIR and NIR, the 5 factors from the PARAFAC model of EEM data, and the peak areas of the 62 resolved components by MCR of ^{1}H-NMR data.

As in the case of low-level fusion, since the extracted features in mid-level data fusion can have different numerical characteristics, scaling of the fused matrix [2,15,17] was performed. Different preprocessing tools were assessed: autoscaling and block-autoscaling (each data set corresponding to an analytical technique was considered as a block). In block-autoscaling, each variable is first scaled to unit variance (autoscaling), and then each block is scaled to equal variance. As a result, each block presented unit variance and each variable inside a block had its variance equal to $1/n_{\text{block}}$, where n_{block} is the number of variables in a given block.

2.3.5.3. P-ComDim. The recently proposed P-ComDim (*i.e.,* Predictive ComDim) method [24], which is the extension of the multiblock method ComDim to the supervised context, has also been evaluated as a different data fusion strategy. For details on P-ComDim algorithm the reader is referred to literature [24,25]. Briefly we recall the main feature of the method. P-ComDim can be applied to any number of data blocks, of which the dependent one is denoted by $\boldsymbol{Y}$ and the independent ones by $\boldsymbol{X}_k$. The first step in P-ComDim algorithm is calculating the kernel matrices:

$$\boldsymbol{S}_k = \boldsymbol{X}_k \boldsymbol{X}_k^{\mathbf{T}} \boldsymbol{Y}\boldsymbol{Y}^{\mathbf{T}} \quad (1)$$

Then a "common singular value decomposition" is conducted, by minimizing the criterion:

$$\sum_{k=1}^{K} \|\mathbf{S}_k - \lambda_k \mathbf{t}\mathbf{u}^{\mathrm{T}}\|^2 \quad (2)$$

obtaining a first common component for the **X**-blocks, i.e. $\mathbf{t}_1$, as well as a component in **Y**-space, i.e. $\mathbf{u}_1$. Further components are then calculated sequentially after deflation of both **X**-blocks and **Y**-block. As for standard ComDim, each single **X**-block ($\boldsymbol{X}_k$) contributes to a common component according to its salience, λ_k [29]. It is also possible to associate to each block $\boldsymbol{X}_k$ a local component by calculating:

$$\mathbf{t}^{(k)} = \mathbf{X}_k \mathbf{X}_k^{\mathrm{T}} \mathbf{t} \quad (3)$$

i.e. Eq. (3) maps t into a latent variable which lies in the space spanned by the variables in X_k. This latent variable $t^{(k)}$ is used to recover and interpret the specific contribution of the X_k-block variables to the global latent variable t.

To accomplish classification, the **Y**-block holds the class membership information, as described in Section 2.3.3. and a classification model can be built by applying PLS-DA to the **u**-scores obtained by P-ComDim. Prediction is accomplished by first estimating, in prediction, the **u**-scores for the test samples (**u**-test) in P-ComDim, then using the **u**-test in PLS-DA as prediction set. In our case, the number of PLS-DA components was estimated according to minimum classification error in CV using the same splits and classification rule as described in Section 2.3.3. Also the subdivision in training and test sets was the same as described in Section 2.3.1.

Moreover, in P-ComDim methodology, two different strategies were performed and compared. In the first, ComDim was developed using the raw spectra of MIR, NIR, EEM and ^{1}H-NMR as X-blocks after applying the same spectral preprocessing as described in Section 2.3.4. MIR and NIR data were mean centered, the ^{1}H-NMR data was block-scaled by dividing the spectra into six regions (0.84–1.15, 1.15–1.5, 1.5–2.0, 2.0–2.25, 2.25–3.2, 3.2–9.8 ppm) to compensate for major differences in spectral region signal intensities and the EEM data array of dimensions I-samples × J-excitation × L-emission wavelengths, was unfolded to a matrix of dimensions $I \times JL$.

In the second, the extracted features of each data block (PCA scores from MIR/NIR, MCR peaks areas of resolved components, and PARAFAC factors) were used as $\boldsymbol{X}$-blocks.

Both in the first and second cases, each data table $\boldsymbol{X}_k$ was normalized in order to obtain the data tables having the same inertia as usually done in ComDim algorithm [56].

The interpretation of each model and comparison of two approaches (i.e. with raw spectra and with the features) was performed by studying the saliences, global and local scores/loadings [28,29], and the classification performance.

2.3.6. Software

Preprocessing, PARAFAC, PCA, PLS-DA and NPLS-DA models were calculated by using routines of PLS Toolbox 6.5 (Eigenvector Research Inc.,WA, USA) working under MATLAB environment v.2016a (Mathworks, MA, USA). LDA was calculated by using the Statistics and Machine Learning Toolbox v. 10.1. Multivariate curve resolution was carried out by using the MCR-ALS GUI (http://www.mcrals.info) and a MATLAB routine implemented to automatically work on spectral intervals, courtesy from Prof. R. Bro's group. ^{1}H-NMR data acquisition, Fourier transformation and spectral preprocessing were carried out using Bruker TopSpin 3.0 and Chenomx NMR Suite 7.0 (Chenomx, Edmonton, Canada) was used to obtain a tentative assignment of the ^{1}H-NMR resolved components.

P-ComDim models were obtained by using routines developed by Prof. D. Rutledge and the SAISIR package for MATLAB [57,58].

3. Results and discussion

This section is articulated in three main parts. In the first one, the description of exploratory analysis results for the individual data sets, as well as the feature extraction step (Section 3.1), and the respective classification models (Section 3.2) are reported. In the second part

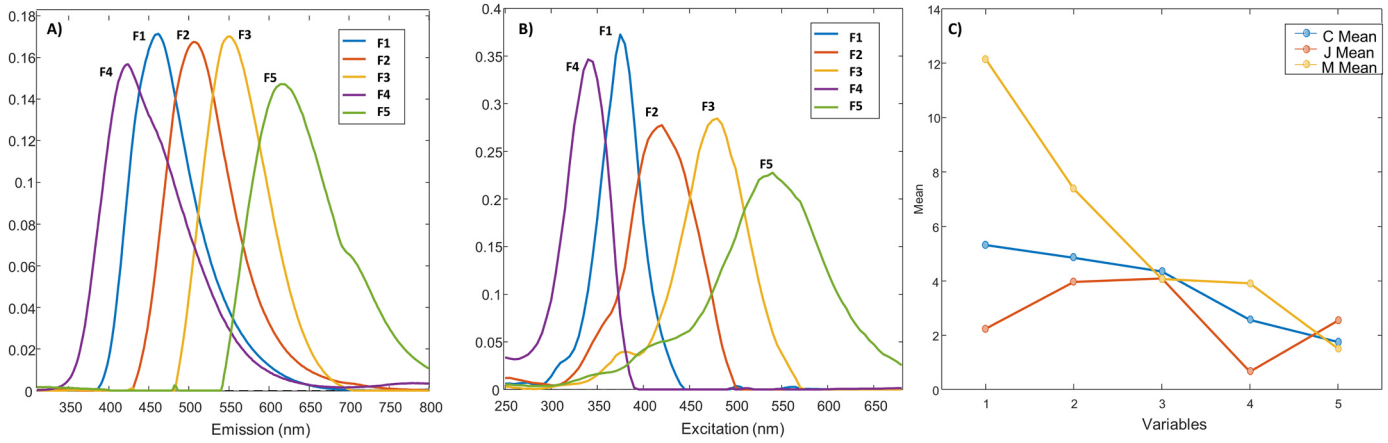

Fig. 2. Emission and Excitation spectra (PARAFAC loadings) of the main fluorophores present in the PDO wine vinegars (A and B). Mean PARAFAC scores of each PDO for the five resolved components (C). The acronyms for the different vinegar PDOs are defined in Table 1.

(Section 3.3), the fused dataset is considered and the application of the mid-level approach is described in detail. The third part (Section 3.4) presents the results obtained by P-ComDim in order to study the complementarity of the techniques.

1. Exploratory analysis of individual data matrices

MIR and NIR data were preprocessed and fused as described in Section 2.3.5.1, the results of exploratory PCA analysis (8 PCs, accounting for 99.8% of the total variance) are reported in Figure III of the Supplementary Material. The three categories strongly overlap and a partial trend of separation was only observed on the scores plot of the PC1, PC3 and PC8 (Fig. III.A), inspecting the corresponding loading plots (Fig. III.B) it can be observed that PC1 mainly distinguishes the sweet Pedro Ximenez sub-category which is present in both "Vinagre de Jerez" and "Vinagre de Montilla-Moriles" PDOs (the contributing spectral regions have been associated with the presence of grape sugars, furfural and Maillard compounds [6,12,46,59]). "Vinagre de Montilla-Moriles" PDO samples are partially separated from "Vinagre de Jerez" PDO along PC3 to which are contributing peaks (Fig. III.B) that have been assigned to chemical compounds that change during aging, e.g. some alcohol, aldehydes, esters, ethers and acids [6,12,46,60,61].

The EEM data array was preprocessed and decomposed by PARAFAC as described in Section 2.3.4.2 obtaining a five factors model (explained variance 99%), which is in good agreement with the three individual PARAFAC models obtained in our previous work [7] for each one of the three PDOs. Fig. 2.A and B includes the PARAFAC loadings for mode 2 and 3 (excitation and emission spectra) of the extracted factors. The excitation and emission maxima of these extracted factors, as well as their possible matching fluorophores according to the literature and our previous knowledge [62-68], are listed in Table 2.

Fig. 3.C shows the average value of the scores (first mode loadings) for samples belonging to each PDO vs. the number of PARAFAC factors. The "Vinagre de Montilla-Moriles" PDO presents higher values on the first and the second factors, with respect to the other two PDOs. Hence, higher presence of components coming from raw materials, which is indicative of less aging, as well as more amount of caramel and 5-Hydroxymethylfurfural (Table 2). However, it is difficult to highlight a clear separation of samples belonging to each class in any of the scatter plots of PARAFAC scores (plots not shown for sake of brevity).

The NMR data set built with the integrated areas of the sixty-two resolved components (Table 3), obtained by MCR analysis of the ^{1}H-NMR spectra (as is detailed in Section 2.3.3.4) was preprocessed by autoscaling prior to PCA analysis (six components, explained variance 90.3%). The score and loading plots of the PCs that better highlighted the separation between the three PDOs are shown on Figure IV Supplementary Material. Also in this case a strong overlap is present and only a partial separation trend of "Vinagre de Montilla-Moriles" PDO samples from "Vinagre de Condado de Huelva" can be observed. The loadings plot (Fig. IVB) highlight, similar to MIR-NIR PCA results, that: i) the first component distinguishes the Pedro Ximenez sweet samples from the rest (contribution from the sugar spectral region, compounds labeled from 34 to 43 in Table 3) and ii) samples from "Vinagre de Condado de Huelva" PDO seem to have higher amount of acetic acid (feature named 18 in Table 3) and ethanol (features 8 and 37, Table 3 with respect to the other two PDOs (separation on PC5).

3.1. Classification results of individual datasets

In a first stage, separate classification models (PLS-DA for MIR + NIR and ^{1}H-NMR data sets and NPLS-DA as described in previous sections for EEM data) were built on the data coming from the different instrumental techniques. The distinct datasets were split in the same training and test sets of 47 and 19 samples as described in Section 2.3.

The classification results obtained by the application of PLS-DA and NPLS-DA on each separate data set, according to the classification criterion described in Section 2.3.3, are reported in Table 4, which reports for each spectroscopic technique the data preprocessing, the model dimensionality (assessed by cross-validation) and the classification performance. PLS-DA was built on the PCA scores (8 PCs) for the MIR-NIR data set, and on the sixty-two peak areas of MCR resolved components for the NMR data set, respectively. While for EEM data set, NPLS-DA was directly built on the spectral data array (samples x excitation wavelengths x emission wavelengths).

The classification results, in calibration, are promising for ^{1}H-NMR models (correct classification rates higher than 90% for all categories).

Table 2
Emission and Excitation maxima of the 5 factor PARAFAC model and their possible matching fluorophores.

	F1	F2	F3	F4	F5
Ex/Em (nm)	**380/450**	**425/520**	**475/565**	**380/425**	**550/630**
Fluorophores	Cumarins, tannins, phenols, flavonols from wine	5-Hydroxmethylfurfural caramel	Vitamin B2 and its principal forms	Phenolic compounds, Maillard products, oxidation products	Unknown related to Pedro Ximenez vinegars

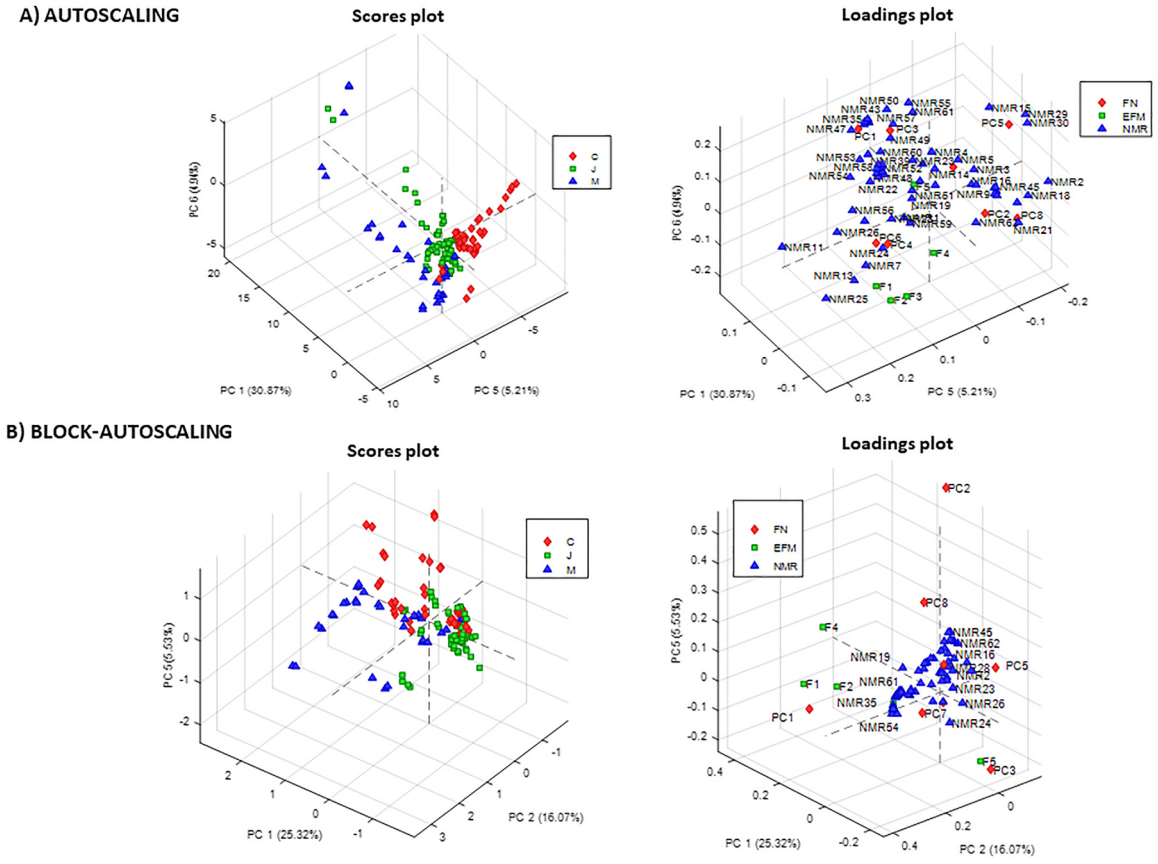

Fig. 3. 3-D plot of PCA scores and loadings obtained for both data fusion strategies (with autoscaling and block-autoscaling preprocessing). The acronyms for the different vinegar PDOs are defined in Table 1.

The model dimensionality, i.e. 7 components, is lower with respect to MIR + NIR, i.e. 10, and EEM, i.e. 12, probably because in this case peak areas of resolved spectral components are used instead of the spectroscopic signal itself. In contrast, the models built on MIR + NIR data and EEM show quite good classification rates, only for one of the category, namely "Condado" and "Jerez" for MIR + NIR and EEM, respectively. These results agree with what already observed in our previous studies [6,7], in which it was shown that these techniques had a better ability to distinguish between categories (aging and sweet) than among the different PDOs.

It can also be observed, that NPLS-DA requires an higher number of latent variables, with respect to the number of PARAFAC factors obtained for EEM data (i.e. five), this could be explained by the fact that NPLS-DA (as PLS usually does) modulates the main fluorophores present in the matrix as well as the environment effects ant the interferences.

On the other hand, the predictive capability (external validation) was almost similar for all the techniques. In general, the results could be considered fairly good, taking into account that, due to the limited number of test samples, for example, in the case of ^{1}H-NMR, 75% correct prediction rates for the classes "Vinagre de Jerez" and "Vinagre de Montilla-Moriles" PDO correspond to 2 and 1 misclassified samples, respectively. In all the prediction models, the same sample of "Vinagre de Jerez" PDO sample was misclassified; also one sample of "Vinagre de Montilla-Moriles" PDO was always misclassified.

Furthermore, it can be observed that prediction rates were higher for "Vinagre de Condado de Huelva" (MIR-NIR and ^{1}H-NMR models) and "Vinagre de Jerez" (EEM model) with respect to "Vinagre de Montilla-Moriles". This fact could be mainly explained by the relative new recognition of this PDO (included in the European Register of Protected Geographical Indications and Protected Designation (PGI) in 2015), in comparison with the other two PDOs, "Vinagre de Condado de Huelva" and "Vinagre de Jerez" PDOs, and specially the last one that was the first wine vinegar PDO of Spain [3]. Furthermore, this is in agreement with our previous studies [7].

To summarize, even though the results are quite promising, the quality of each model was not enough good for the characterization and classification purpose and it varied significantly from one technique to another.

3.2. Mid-level data fusion

The results described in Sections 3.1.4 showed that classification models built on each of the individual data matrices are not accurate enough, indicating that a single instrumental fingerprint is not completely able to correctly predict the high-complex samples under study. For this reason, the possibility of combining the information from the different instruments by means of mid-level data fusion strategy was investigated.

The features obtained from the decomposition of the single data blocks (i.e. the eight MIR + NIR PCA scores, the five factors EEM PARAFAC scores and the peak areas of the sixty-two resolved ^{1}H-NMR MCR components) were merged in a unique block as described in Section 2.3.4 (Fig. 1). Since scaling is a critical issue both block-autoscaling and autoscaling (Section 2.3.4.) were compared.

Explorative PCA models were built with the fused data preprocessed by both scaling's methods and results shown in Fig. 3. The autoscaled data (Fig. 3.A) showed a similar clustering of the three PDOs as the one observed in the score plot of ^{1}H-NMR PCA reported in Fig. IV.A Supplementary Material. In particular, PC1 distinguish the samples belonging to the sweet category at positive values of PC1. "Vinagre de Montilla-Moriles" PDO showed positive scores values on PC5, whereas

Table 3
MCR resolved, integrated and interpreted components for ^{1}H-NMR data.

RT	Type[a]	Code	Interpretation
0.86–0.9	t	NMR1	2-Hydorxy-3-methylvalerate
0.9–0.97	d + m	NMR2	X1
0.98–1.02	t + q	NMR3	X3
	–	NMR4	X4
1.03–1.06	d	NMR5	Isobutyrate
1.06–1.11	t	NMR6	Propionate
1.11–1.16	d	NMR7	Isopropanol
1.17–1.20	t	NMR8	Ethanol
1.22–1.29	q	NMR9	X5
1.30–1.34	d + q	NMR10	X6
1.35–1.38	d	NMR11	Acetoin
1.39–1.43	d	NMR12	Lactate/2-Phenylpropionate
1.48–1.53	s + t	NMR13	X7
	–	NMR14,NMR15	X8, X9
1.77–1.81	q	NMR16	6-Acetylglucose
1.97–2.00	s	NMR17	Acetamide
2.02–2.12	s	NMR18	Acetic Acid
2.12–2.14	s/d	NMR19	X10
2.13–2.16	s/d	NMR20	X11
2.16–2.19	s	NMR21	Acetoin
2.21–2.25	s + d	NMR22	Acetone
	dd	NMR23	Acetone
2.28–2.30	s	NMR24	Acetoacetate,Acetylsalicilate
2.32–2.34	d	NMR25	X12
2.37–2.40	s + t	NMR26	Malate, Glutarate, N-Acetylglutamate…
2.59–2.62	t	NMR27	Beta-Alanine, Succinate…
2.64–2.67	s	NMR28	Succinic Acid
2.81–2.85	d	NMR29	X13
2.96–3.01	d	NMR30	X14
3.18–3.21	s	NMR31	Acetylcholine
3.22–3.31	m	NMR32	Glucose
3.30–3.36	d	NMR33	Methanol
3.37–3.51	m + m	NMR34	Glucose
3.51–3.58	m	NMR35	Glucose
3.57–3.65	d	NMR36	Glucose + Fructose
3.63–3.67	q	NMR37	Ethanol
3.67–3.74	m	NMR38	Fructose + Glucose
3.74–3.78	dd	NMR39	Glucose
3.78–3.84	m	NMR40	Fructose
3.84–3.86	d	NMR41	X15
3.87–3.91	dd	NMR42	Fructose + Glucose
3.98–4.03	d + s	NMR43	Frcutose
4.09–4.12	t	NMR44	Frcutose
4.11–4.15	q	NMR45	X17
4.51–4.54	d + s	NMR46	X20
4.56–4.60	d	NMR47	X21
4.62–4.68	d	NMR48	Glucose
4.68–4.71	s	NMR49,NMR50,NMR51	5-HMF
5.21–5.26	d	NMR52	Glucose
5.35–5.39	d	NMR53	X22
	–	NMR54	X23
6.67–6.70	d	NMR55	X24
	–	NMR56	X25
7.52–7.55	d	NMR57	X26
8.25–8.28	s	NMR58, NMR59	Formic Acid
9.43–9.47	s	NMR60, NMR61	5-HMF
9.65–9.68	q	NMR62	X27

[a] Peak multiplicities: s, singlet; d, doublet; t, triplet; dd, doublet of doublets; q, quadruplet; m, multiplet.

"Vinagre de Condado de Huelva" PDO samples showed negative scores values for this component and samples of "Vinagre de Jerez" PDO are placed again in the middle. Fig. 3.B shows the loading plot of the same principal components, in which the contribution of several of the features, both from ^{1}H-NMR and MIR-NIR was observed. PC5, PC2 and PC8 from MIR-NIR PCA, as well as several of the NMR features, seem to be the main responsible features for the improvement in the separation of "Vinagre de Condado de Huelva" and "Vinagre de Montilla-Moriles" samples. In fact, they have high negative loadings values on the fifth component of the PCA on fused data, while at positive loadings values there are PC4 and PC6 from MIR-NIR PCA and F1-F3 from PARAFAC. PC1 from MIR-NIR PCA seems of relevance in the Pedro Ximenez samples separation from the rest, since its high positive loadings on the first component of the PCA on fused data.

Even if few minor differences were noticed with respect to ^{1}H-NMR data analysis, some improvements in the separation of PDOs occurred. The similarity between the fused autoscaled data and the ^{1}H-NMR data block is explained by the fact that using autoscaling as merging strategy, a higher importance is given to the block of variables more numerous, hence, the ^{1}H-NMR data.

Regarding the block-autoscaling PCA results (Fig. 3B), the principal components that better shows a separation were PC1, PC2 and PC5. In this scores plot, the separation of PDOs seems to be worse than with autoscaling procedure. Thus, a higher overlapping between "Vinagre de Jerez" and "Vinagre de Condado de Huelva" samples was observed. In spite of this, "Vinagre de Jerez" PDO seems to be mainly placed in the negative side of PC1 while "Vinagre de Condado de Huelva" in the positive side of PC1 and PC5, and "Vinagre de Montilla-Moriles" PDO in the positive side of PC2. The loadings plot (Fig. 3.D) shows in this particular scaling procedure that ^{1}H-NMR components had lower relevance and the MIR-NIR and EEM variables became more influential. Thus, PC3 (MIR-NIR) and F5 (EEM) showed the most negative contribution of PC1, while F4, F1 and PC1 the most positive, as well as PC2 and PC8 of MIR-NIR data had the most positive values of PC5, relevant for the separation of "Vinagre de Condado de Huelva" PDO.

Then, PLS-DA models were built using six and seven latent variables for autoscaling and block-autoscaling procedures, respectively (chosen accordingly to minimum cross validation classification errors). The results obtained are reported in Table 5. They confirmed the improvement with respect to the classification models obtained for the separate data blocks. In fact, 100% of correct classification was obtained for the predicted samples (test set) of all the PDOs, as well as 100% of "Vinagre de Condado de Huelva" PDO samples were correctly classified in both fit and the prediction. The two scaling procedures give very similar PLS-DA classification rates, only the number of latent variables were different.

In order to identify the most effective variables in discriminating the PDO samples, the values of the PLS-DA regression vectors and the variable importance in projection (VIP) index were studied; for interpretative purposes all the predictors having a VIP > 1 are considered to be relevant [69]. Despite the different scaling procedure, the variables with VIP higher than one quite matched in both PLS-DA models and are reported in Table 6 together with the sign of the corresponding regression coefficients. Accordingly, the most relevant variables for the discrimination of the "Vinagre de Condado de Huelva" PDO were mainly MIR-NIR PC2, PC3, PC5 and PC8 previously described as the spectral regions related to the presence of acetic acid and ethanol (~1410 and ~1290 cm^{-1} and 1045 cm^{-1} in MIR spectra) as well as alcohol compounds, aldehydes, and some esters and ethers that matched with PC3 loadings. Other important variables were EFM F1 and F4 that matched with the presence of phenolic compounds and NMR7, NMR11, NMR16, NMR18 and NMR27 that were interpreted as isopropanol, acetic acid, acetoin and some other compounds such as 6-acetylglucose, beta-alanine and succinates.

Regarding "Vinagre de Jerez" PDO, this PDO was described mostly by the variables PC3 and PC8 of MIR-NIR PCA, related to alcohol compounds, aldehydes, esters, ethers and acids and commonly presented in grapes, wine and vinegar; EEM F5 related to grape sugars, furfural and Maillard compounds more presented the Pedro Ximenez category included in this PDO, together with F1 and F4 again; and NMR16, NMR26 and NMR59 identified as 6-acetylglucose, aminoacids as malate, glutarate or n-acetylglutamate and formic acid, respectively.

Finally, the variables that seems to give a relevant contribution for the classification of "Vinagre de Montilla-Moriles" PDO were mainly: MIR-NIR PC5 and PC8 whose loadings mainly showed a peak at 1045 cm^{-1} and PC1 again related to the Pedro Ximenez samples of this

Table 4
Classification results for each individual data block.

Data	Classification method	Pretreatment	LV[a]	% Corrected clasified					
				Train[b]			Test[b]		
				C	J	M	C	J	M
MIR + NIR	PLS-DA	Block Scaling + Mean Centering	10	90.0	85.0	79.2	100	87.5	62.5
EEM	NPLS-DA	Mean centering	12	66.7	95.0	75.0	50.0	100	83.3
^{1}H-NMR peak areas	PLS-DA	Autoscaling	7	100	97.5	91.7	100	75.0	75.0

[a] LVs number determined on the basis of minimum classification error in CV (Venetian blind 7 splits, keeping replicates in the same set).
[b] Independent train and test sets, average correct classification rate for 5 random training/ test splitting is reported.

Table 5
PLS-DA results obtained by mid-level fused dataset with two different scaling procedures.

Dataset	Classification method	Pretreatment	LVs[a]	% Corrected classified					
				Train[b]			Test[b]		
				C	J	M	C	J	M
Mid-Level Data Fusion	PLSDA	Autoscaling	6	100	100	91.7	100	100	100
		Block-Autoscaling	7	100	97.5	91.7	100	100	100
P-Comdim Raw		Autoscaling	2	90.0	97.5	75.0	50.0	75.0	75.0
P-Comdim Extracted Features		Autoscaling	2	96.7	100	87.5	91.7	87.5	87.5

[a] LVs number determined on the basis of minimum RMSECV with Venetian blind cross validation (7 splits, 2 samples per split).
[b] Independent test set, average correct classification rate for 5 random training/ test splitting is reported.

PDO; EFM F1 and F5, which brings mainly the information of the compounds commonly presented in grapes and wine such as cumarins, tannins, phenols, flavonols, and moreover, compounds related to the sweet category such as HMF and sugars also related to the NMR most relevant variables according to the VIPs (i.e. compounds from NMR32 to NMR52). These results agree with those obtained in the loadings plot of the PCA model previously described (Fig. 3).

3.3. P-ComDim

P-ComDim was carried out with the raw spectral data (Fig. 4a and Fig. 5a) and the data of the extracted features (Fig. 4b and Fig. 5b) in order to study the best approach that show the complementarity of the techniques and therefore also their differences. Fig. 4 and Fig. 5 shows the saliences and the global loadings obtained [28,70] for each technique, respectively.

In Fig. 4 on the top is shown the percentage of variance extracted by each common component (graph on top left), the sum of saliences of all data tables for each common component (graph on top middle) and the sum of saliences for each data table over all the calculated common components (graph on top right). Taking into account the normalization of the single data table, the sum of saliences in the latter plot can be at maximum equal to 1, when no residual variance is left, for that data table after extracting the common components. In the bottom part of Fig. 4 are shown the saliences of each data table on each common component. The sum of the saliences reported on top of each graph corresponds to the values reported on the top middle graph. The first two components explain most of the data variance but taking into account eight components allows describing all data tables.

The analysis of salience for the raw spectral data (Fig. 4.A) show that MIR and NIR share mainly one common component, i.e. CC1, while EEM and ^{1}H-NMR data seem to capture most distinctive information, contributing to different components, namely CC2 for EEM and CC3, CC4, CC5 and CC6 for ^{1}H-NMR. Despite with lower weights, CC8 is common to MIR, NIR and ^{1}H-NMR data blocks and CC7 to all of them. Regarding the loadings vectors associated to each block (Fig. 5. A), CC1 seemed to be related to the Pedro Ximenez category due to the intense band showed in MIR and NIR loadings plot (between 1000 and 1150 cm-1 and 5200 and 6500 cm^{-1}, respectively) and in ^{1}H-NMR data point to a higher intensity in the sugar region of the spectra (from 3.22 to 4.12 ppm); CC2, considering the excitation and emission wavelengths of the EEM reshaped landscapes, resemble the first PARAFAC factor (Fig. 2), while CC3 was related to the first region of the ^{1}H-NMR spectra were acids (e.g. acetic acid), alcohols (e.g. ethanol) and some esters (isobutyrate) appear. Finally, CC7 seemed to be associated to the presence of acetic acid and ethanol that could be observed by NIR, MIR

Table 6
Salient variables for discrimination for each PDO category according to PLS-DA VIP values, which were concordant in both DF PLS-DA models, i.e. autoscaling and block-autoscaling. In parenthesis, the sign of the corresponding regression coefficients is reported.

PDOs	NIR-MIR	^{1}H-NMR	EEM
"Vinagre de Condado de Huelva"	PC2(+), PC3(-), PC5(+), PC8 (+)	NMR7(-), NMR11(-), NMR16(+), NMR17(+), NMR18(+), NMR24(-), NMR26(-), NMR27(+), NMR29(+), NMR30(+), NMR31(-)	F1(-),F4(+), F5(+)
"Vinagre de Jerez"	PC1(-), PC2(-), PC3 (+), PC4(-), PC7(-), PC8(-)	NMR14(+), NMR16(-), NMR26(+), NMR27(-), NMR29(-), NMR31(+), NMR59(-)	F1(-), F4(-), F5(+)
"Vinagre de Montilla-Moriles"	PC1(+), PC5(-), PC8(-)	NMR16(-), NMR26(-), NMR27(-), NMR32(+), NMR35(+), NMR36(+), NMR39(+), NMR44(+), NMR48(+), NMR49(+), NMR51(+), NMR59(+), NMR61(+)	F1(+), F5(-)

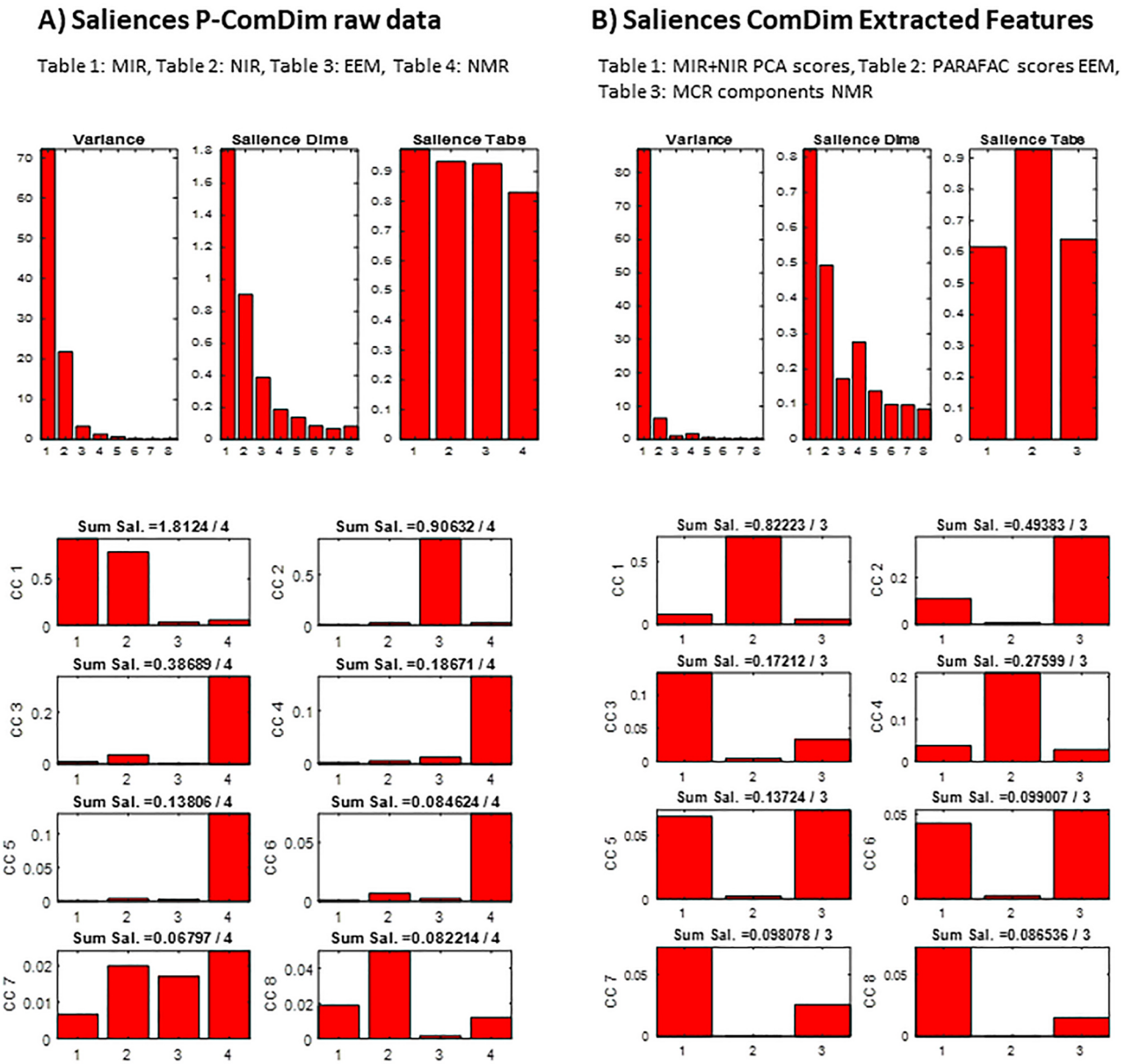

Fig. 4. Graph of saliences and sum of saliences obtained by the P-ComDim method developed with the raw data (A) and with the extracted features (B).

and ^{1}H-NMR techniques and, as far as EEM loadings are concerned, the profile resembles those of the fourth PARAFAC factor which was associated to phenols compounds.

In the case of P-ComDim model, obtained with the extracted features of each data block (Fig. 4. B), EEM (data table numbered as 2 in the figure) has again little in common with the other data tables and mainly contribute to CC1 and CC4, which by inspection of loadings are related to the first four PARAFAC factors (CC1) and second, third and fifth factors (CC3), respectively. ^{1}H-NMR data contribute mainly to CC5 and CC6 together with MIR and NIR data, i.e. these global components are shared by these data tables and, hence, should reflect the samples trends common to ^{1}H-NMR and MIR-NIR. CC2 is mainly contributing the NMR data table and the respective loadings (Fig. 5B) show high influence of the first region of the ^{1}H-NMR spectra (alcohols and acids). CC3, CC7 and CC8 are mainly contributing the MIR-NIR data table, in particular, according to the loadings plot (Fig. 5B), the PC6 and PC8 scores of PCA decomposition of NIR-MIR spectra.

Fig. 6 illustrates the global scores scatter plot obtained by P-ComDim analysis (the bottom plot in Fig. 6A and the bottom right one in Fig. 6B). In comparison to PCA analysis of individual spectral data sets (Figs. II and IV Supplementary Material), ComDim clearly shows an increased separation trend according to the PDO, even though this separation was slightly worse than in the PCA obtained on the mid-level fused data (Fig. 3). These results are consistent with the fact that the global scores scatter plot of P-ComDim obtained on the extracted features data tables, i.e. corresponding to the data used for the mid-level data fusion, show a better separation among PDOs than the ComDim performed on the raw spectral data. These results could be better observed by the scores plot of the PLS-DA models obtained for each approach (Fig. 6). Thus, this latter figure showed that more overlapping occurs when PLS-DA is carried out with raw data than by using the extracted features of each data set (i.e. six samples were not correctly predicted by the raw data model with respect to the two samples wrongly predicted by the model with extracted features). Nonetheless, one advantage of performing P-ComDim directly on the raw spectra is the interpretation of the spectral regions contribution by visualization of the corresponding local loadings.

The classification results expressed as percentage of corrected

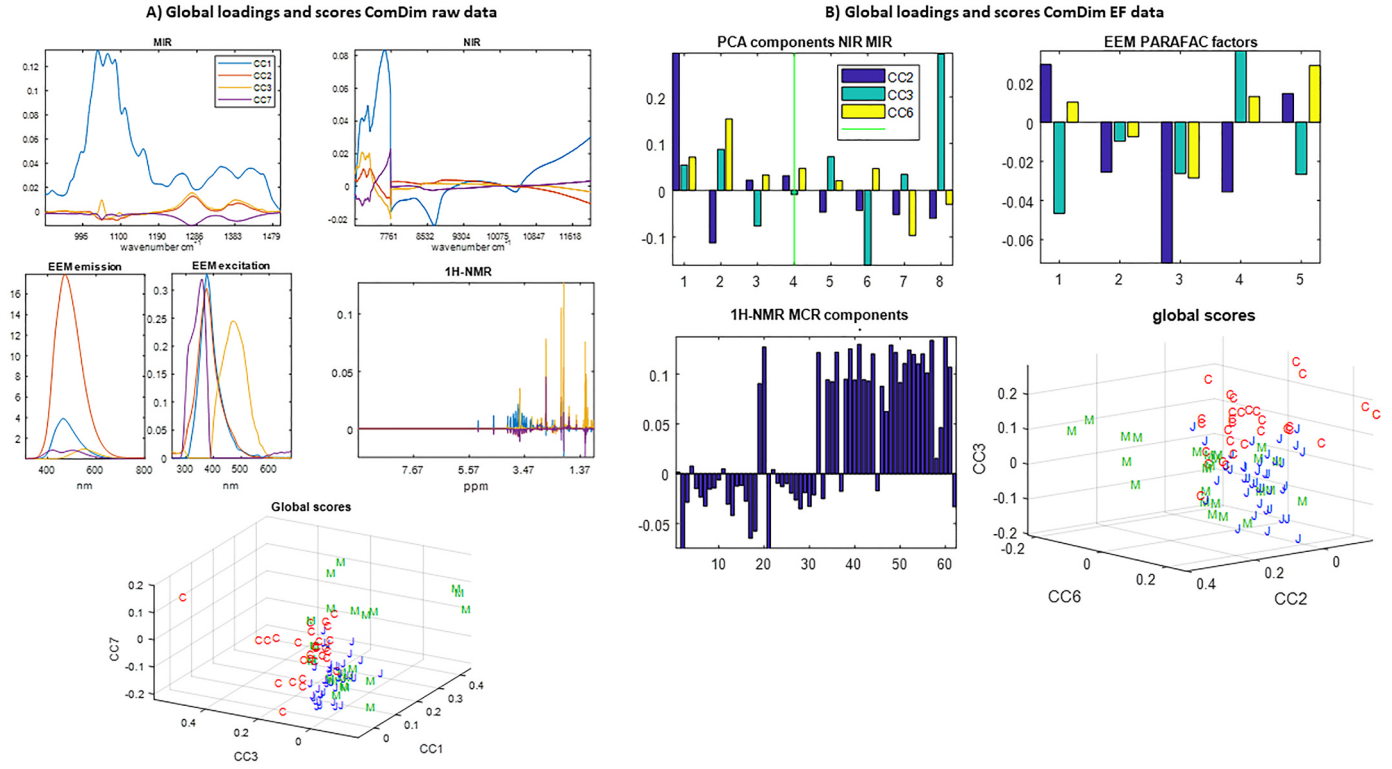

Fig. 5. Global loadings for each data block and global scores plot obtained by P-ComDim method carried out by using the raw spectral data of MIR, NIR, ^{1}H-NMR and EFM scores (A) and the data of extracted features obtained by MIR-NIR PCA, EFM PARAFAC and ^{1}H-NMR MCR compounds (B).

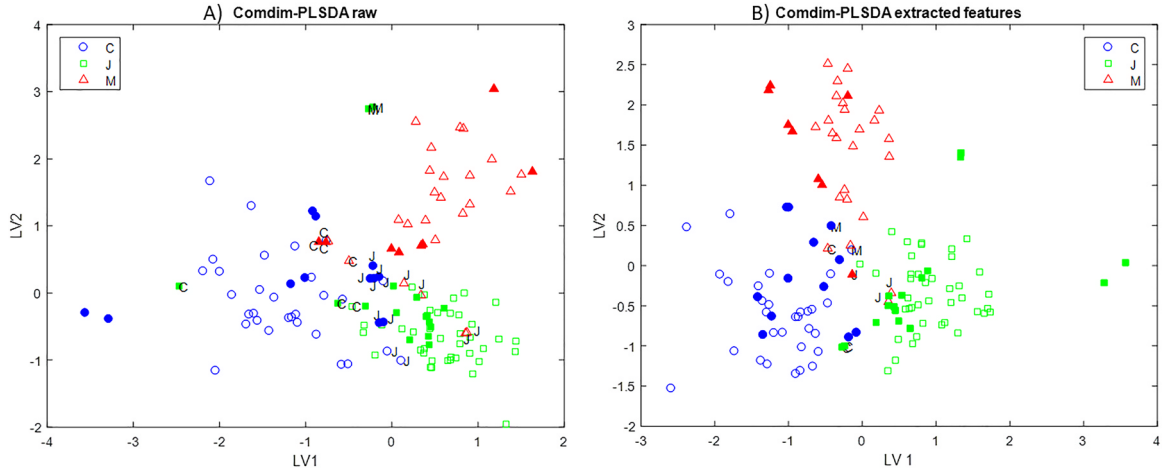

Fig. 6. Scores for the first two latent variables of the PLS-DA classification model obtained by P-ComDim with the raw data (A) and extracted features (B). The acronyms for the different vinegar PDOs are defined in Table 1. Test samples are represented by filled symbols. The labels (letter indicate the category predicted by the model) highlight misclassified samples.

classified by means of PLS-DA model carried out with P-ComDim results are reported in Table 5 together with the classification results obtained by the mid-level data fusion models. Looking at the table it can be noticed once more that the results obtained by the PLSDA performed on the P-ComDim scores from the extracted features were better than the PLS-DA results obtained by each data set individually studied, only comparable to the ^{1}H-NMR results, as well as they were better than the P-ComDim classification model developed with raw data. However, in spite the promising classification rates obtained by the P-ComDim with the extracted features, the classification results were inferior to the results obtained by Mid-level data fusion.

4. Conclusions

This study demonstrates the potential of the combination of four spectroscopic analytical methods (MIR, NIR, EFM and ^{1}H-NMR) when they were combined. The application of data fusion methods improved the characterization and authentication of PDO wine vinegars, providing a more efficient differentiation than the models based on single methods. The obtained results support the approach of combining these methods to achieve synergies for an optimized differentiation of the PDO of wine vinegars. With regard to single analytical methods, especially the classification results of ^{1}H-NMR models were promising. On the other hand, the application of P-ComDim method was useful for describing, in a simple and synthetic manner, the overall spectral

information collected and reveal the complementarity and differences of the spectroscopic techniques, assessing the importance of each technique to each of the common variables. However, for a PDO classification objective, the results of the present work showed that Mid-level data fusion can be the better option in comparison to the classification models obtained by P-ComDim. In spite of this fact, this study presents promising results related to the development of efficient classification models by P-ComDim carried out with the extracted features of spectroscopic data.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.talanta.2019.01.100.

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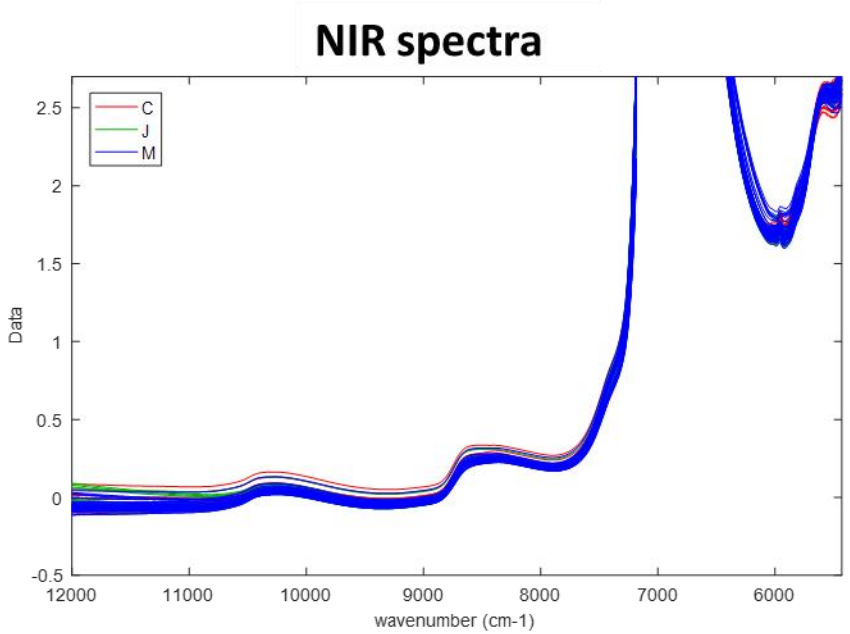
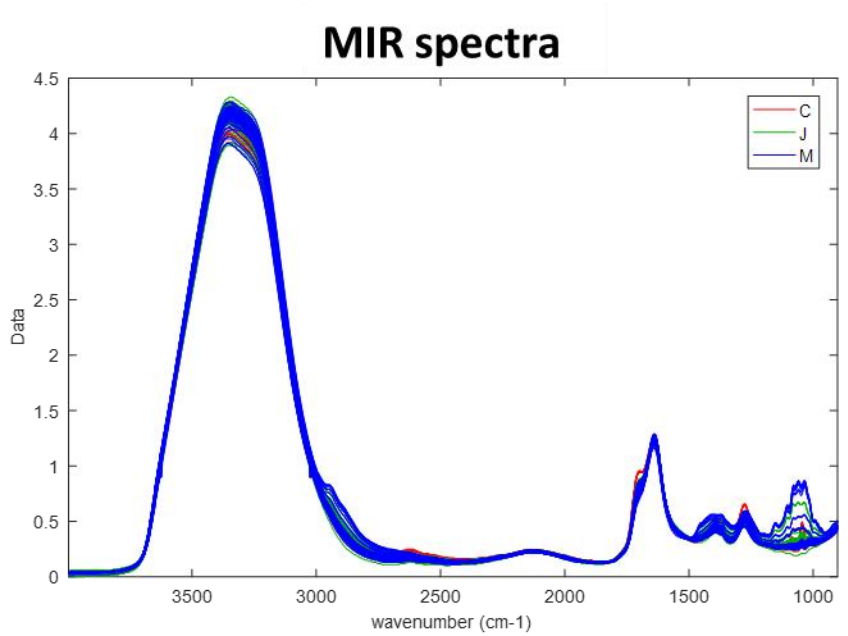
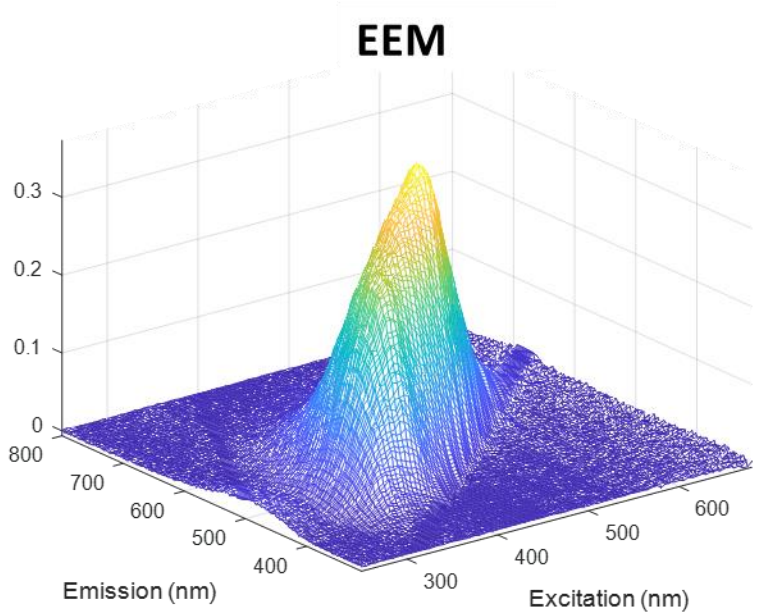
Fig. I







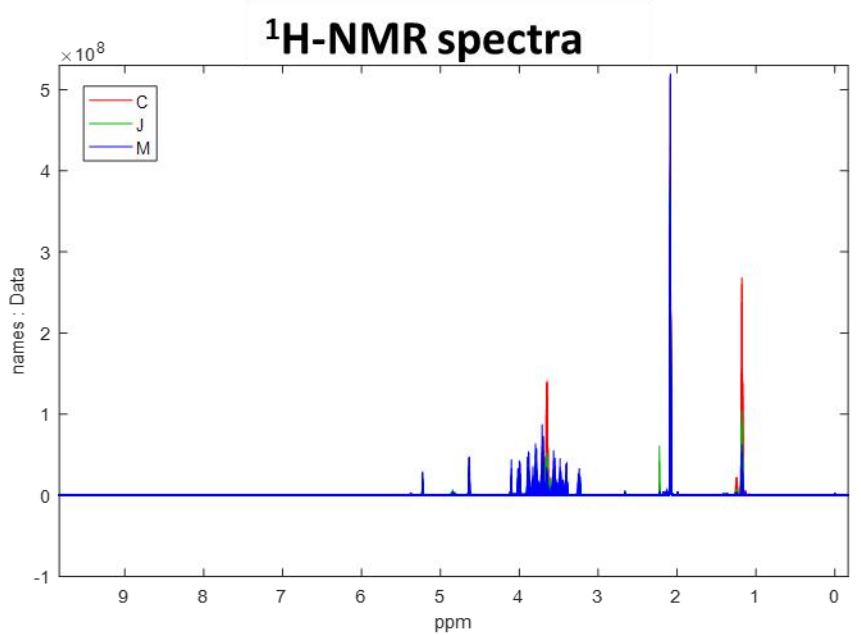


Fig. I. Plot of raw MIR, NIR and ^{1}H-NMR spectra and plot of an example of EEM landscape.

^{1}H-NMR spectra: intervals

Fig. II

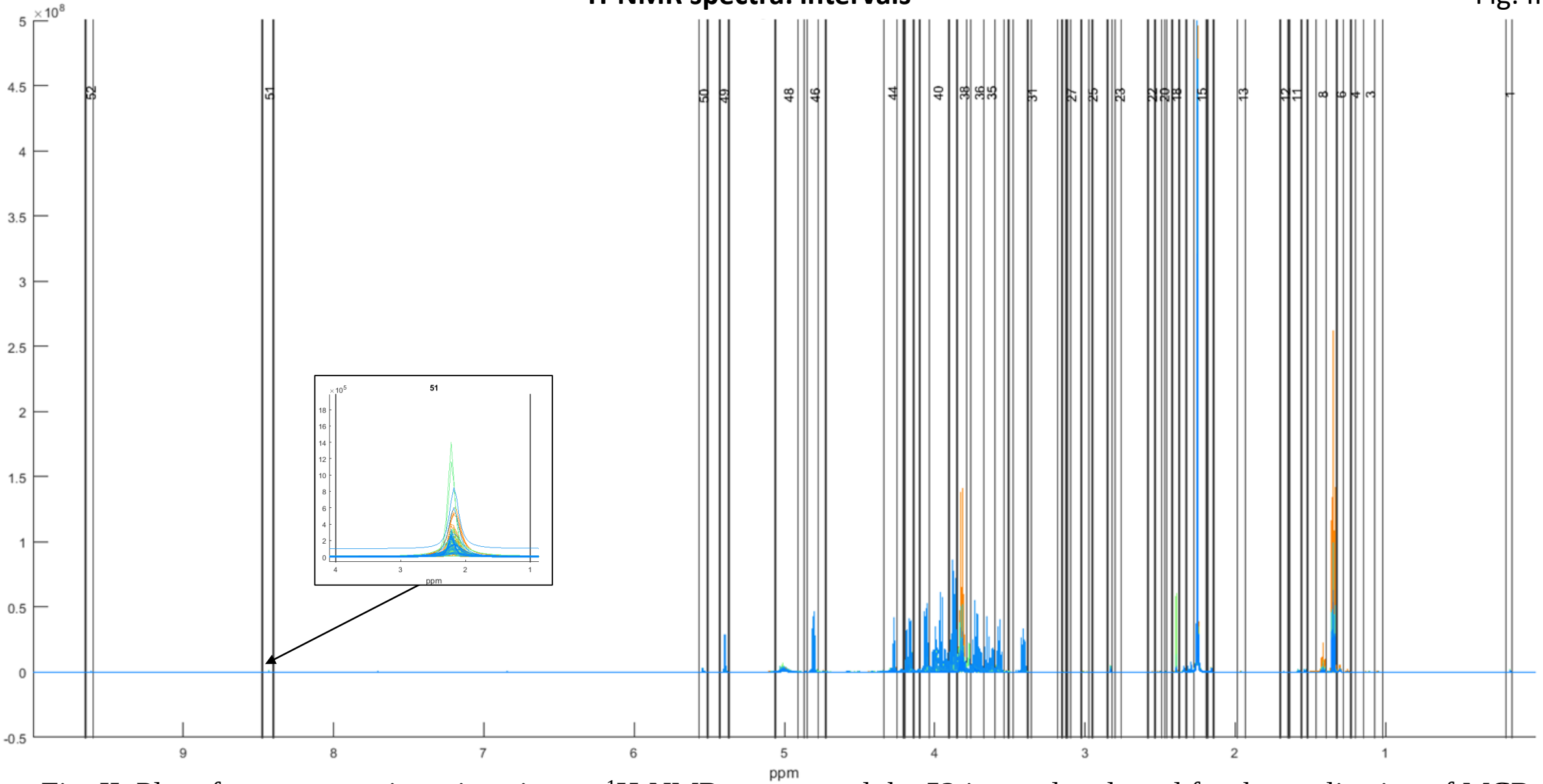


Fig. II. Plot of a representative wine vinegar ^{1}H-NMR spectra and the 52 intervals selected for the application of MCR method. Zoom in interval 51.

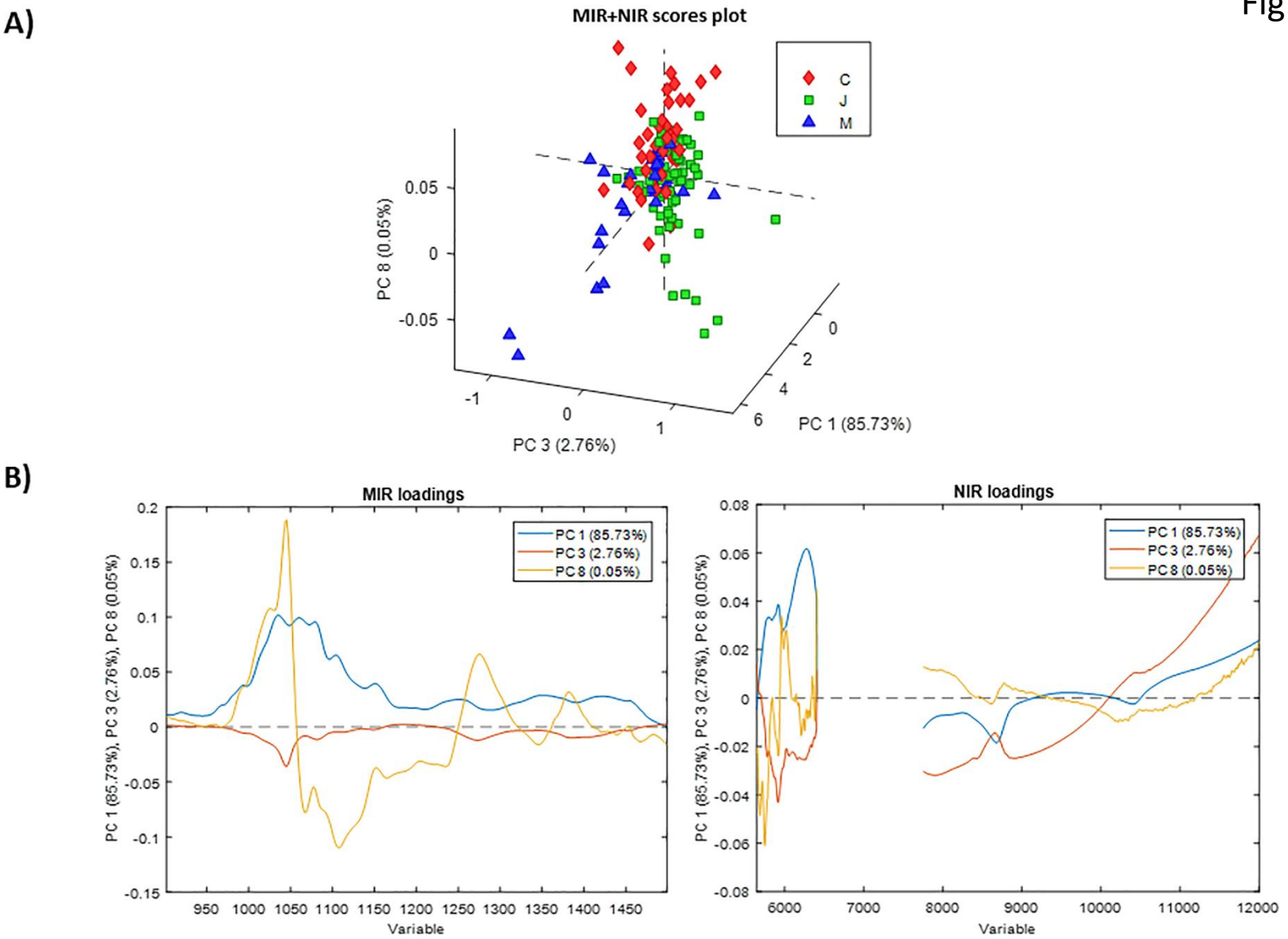


Fig. III. Scores and loadings plot of the PCA model obtained for low level fusion of MIR and NIR data. The acronyms for the different vinegar PDOs are defined in Table 1.

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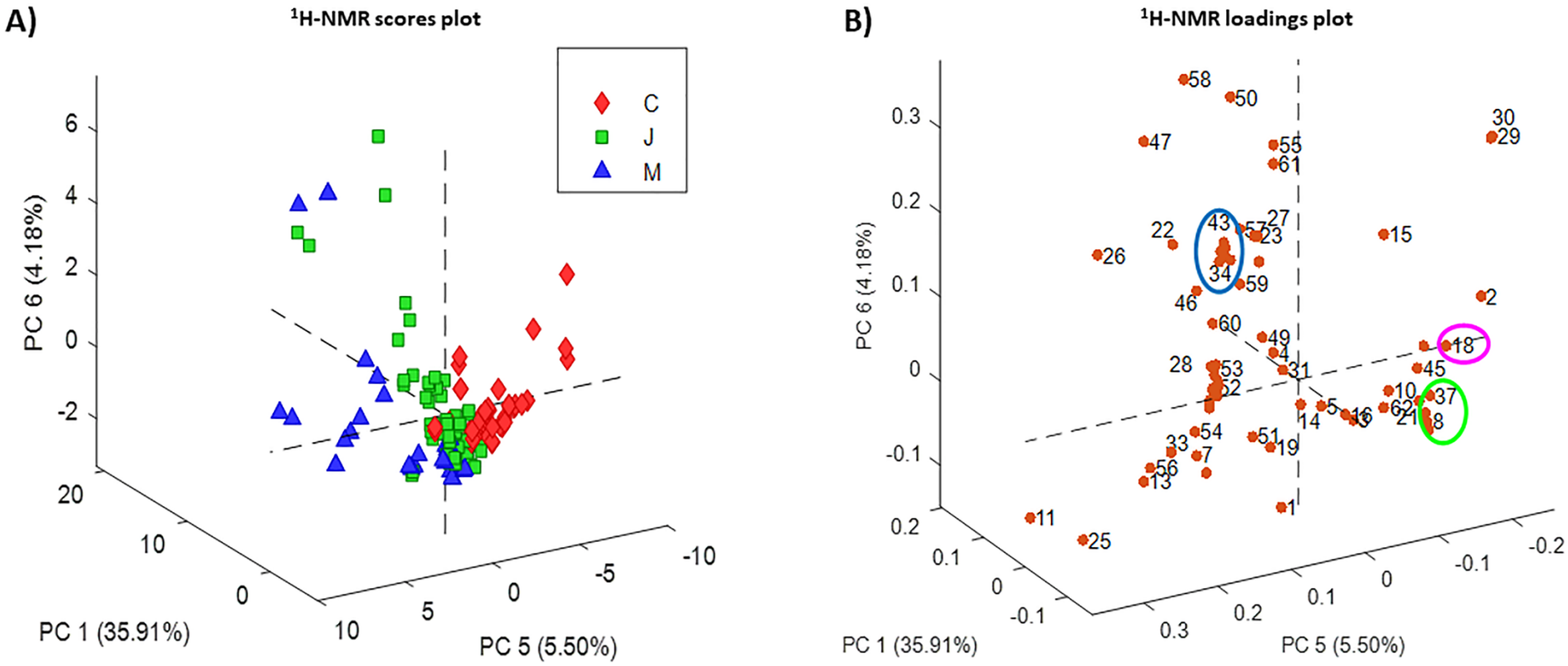

Fig. IV. 3-D Scores and loadings of the PCA model obtained for ^{1}H-NMR data after the application of multivariate curve resolution (MCR) decomposition. The acronyms for the different vinegar PDOs are defined in Table 1.

BLOQUE I:

CARACTERIZACIÓN Y CLASIFICACIÓN ESPECTROSCÓPICA DE LOS VINAGRES DE VINO ESPAÑOLES CON DOP

CAPÍTULO IV.

Espectroscopía de ultravioleta-visible (UV-vis)

CHAPTER IV.

Ultraviolet-visible spectroscopy (UV-vis)

RESUMEN

La espectroscopia ultravioleta-visible (UV-vis), a pesar de ser especialmente atractiva por su simplicidad y bajo costo, y ser una de las técnicas espectroscópicas más simples y económicas del mercado, todavía no había sido utilizada para evaluar la DOP u otras cualidades como el tipo de producción o envejecimiento de los vinagres de vino. Esto fue debido a que la literatura previa sobre el uso de esta técnica en el campo de la clasificación/autentificación de alimentos era escasa y muy reciente, además de que es una técnica que ofrece poca información química de las muestras. Sin embargo, las principales ventajas de esta técnica son su amplia aplicabilidad, rapidez de análisis, ausencia de residuos generados y su facilidad de uso, sin costos ni operador calificado, produciendo resultados con una alta sensibilidad, una selectividad moderada y una buena exactitud. Además, esta técnica presenta la posibilidad de desarrollar dispositivos portátiles de espectroscopía UV para probar la autenticación de algunos alimentos y bebidas.

Por todos estos motivos, en este trabajo, publicado en Chemometrics and Intelligent Laboratory Systems 191 (2019) 42–53, se estudió por primera vez el potencial de la espectroscopia UV-vis con el fin de desarrollar mejores modelos de clasificación de los vinagres de vino según el método de producción, la DOP y la categoría de envejecimiento.

Un total de 70 vinagres de vino se analizaron de manera directa y se compararon los espectros en la región seleccionada de 280-600 nm. Estas muestras estaban formadas por 50 vinagres de vino de las tres DOP de las distintas categorías establecidas, junto con 20 vinagres sin DOP conocidos como “vinagres rápidos” de diferentes orígenes geográficos y distintas calidades. En primer lugar, como en todos los trabajos presentados en los capítulos anteriores, se realizó un análisis de componentes principales (PCA) como método exploratorio, mientras que la clase de modelado independiente suave (SIMCA) y el análisis discriminante de mínimos cuadrados parciales (PLS-DA) se emplearon para el desarrollo de un modelo jerárquico de clasificación (HCM) el cual fue evaluado mediante el método de remuestreo “bootstrap”, que genera distribuciones de resultados de clasificación y permite obtener intervalos de confianza en la clasificación.

Los resultados mostraron que esta metodología era capaz de clasificar o distinguir vinagres envejecidos de no envejecidos, vinagres con DOP de vinagres sin DOP o rápidos, entre diferentes DOPs e incluso entre diferentes categorías de envejecimiento dentro de una DOP, siendo la primera vez que todas las clases se clasifican a la vez con una sola técnica analítica y mediante la combinación de estas metodologías quimiométricas.

Los resultados obtenidos mostraron que la región de los espectros UV alrededor de 300 nm y la región visible entre 500 y 600 nm fueron las responsables de la diferenciación de los vinagres más envejecidos (categoría "Reserva"), dentro de los cuales, a su vez, cada DOP mostró una diferencia en la intensidad y un desplazamiento de estas regiones del espectro. Además, los vinagres de DOP de menor envejecimiento mostraron bandas relevantes del espectro alrededor de 290 nm y entre 350-500 nm, mientras que los vinagres de DOP sin envejecimiento mostraron una intensidad más baja en todo el espectro y el pico principal a 290 nm.

Estos resultados demuestran que, aunque la información contenida en los espectros UV-vis no es específica ya que no se consiguen identificar los compuestos relacionados con el envejecimiento o el origen, esta metodología permite la diferenciación de las categorías y del origen del vinagre de vino, así como de los distintos métodos de producción y DOPs, tanto por la observación directa de los espectros, como por los resultados obtenidos de clasificación.

En conclusión, el modelo de clasificación jerárquica desarrollado en este estudio con espectros UV-vis junto con SIMCA y PLS-DA abre la posibilidad de desarrollar un software que proporcionaría una diferenciación fácil y rápida para la autenticación de vinagres de vino de diferentes categorías y denominaciones de origen. Además, este procedimiento presenta la posibilidad de realizar análisis in situ y no destructivos utilizando un instrumento portátil, pudiendo utilizarse como una herramienta alternativa a los procedimientos de control estándar de los Consejos Reguladores de las tres DOPs.

ARTÍCULO 6

Application of hierarchical classification models and reliability estimation by bootstrapping, for authentication and discrimination of wine vinegars by UV-vis spectroscopy

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Application of hierarchical classification models and reliability estimation by bootstrapping, for authentication and discrimination of wine vinegars by UV–vis spectroscopy

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ABSTRACT

In recent years, three Spanish wine vinegars have obtained the indication of Protected Denomination of Origin (PDOs) due to their unique characteristics and traditional method of production: "Vinagre de Jerez", "Vinagre de Condado de Huelva" and "Vinagre de Montilla-Moriles". These vinegars are expensive due to their high quality, the long aging time and the high cost of production, reason why the adulteration and unfair competition in the vinegar industry are frequent practices. To avoid these frauds, several analytical techniques have been already studied for the characterization and authentication of these high quality vinegars. Nevertheless, ultraviolet–visible (UV–vis) spectroscopy, especially attractive for its simplicity and low cost, has not been previously used to assess PDO or other qualities as type of production or aging, in wine vinegars. For this reason, the potential of UV–vis spectroscopy was investigated for the first time as a rapid and inexpensive methodology for developing classification models to discriminate wine vinegars according to the production method, the PDO and the aging category. Spectra from 70 wine vinegars -including different categories within the 3 PDOs and also vinegars without PDO as known as rapid vinegars-have been analyzed and compared in the selected region of 280–600 nm. Principal components analysis (PCA) was used as exploratory method, while soft independent modelling-class (SIMCA) and partial least squares-discriminant analysis (PLS-DA) were employed for the development of a hierarchical classification model. Differences between categories and PDOs, as well as between PDO and Non-PDO wine vinegars, were observed according to the spectral regions around 300 nm and the visible regions around 500 nm. Furthermore, bootstrap resampling method was employed to generate distributions of classification results and to obtain confidence intervals in the classification. The hierarchical classification results open up the possibility of developing a tool that provides an easy and fast differentiation for the authentication of wine vinegars from different categories and denomination of origins.

1. Introduction

Wine vinegar is a greatly appreciate product in most of the wine producer countries. It is mainly obtained by two different procedures: the submerged method (also called as "rapid" method), and the surface method, which is the traditional and slow one. For the first one, the vinegar is produced in stainless steel accelerators involving submerged cultures, while the second method is performed in wood barrels by using surface cultures. Moreover, the type of wine used for each procedure is usually different. Thus, quality wines are usually used for the "slow" method, while table wines or less quality ones are usually used for the "quick" method, which also has an influence in the quality of the final wine vinegars. Among wine vinegars produced by a slow acetification process, three Spanish wine vinegars have been protected by the indication known as Protected Designation of Origin (PDO). These PDO wine vinegars are "Vinagre de Jerez", "Vinagre de Condado de Huelva" and the most recently accepted, "Vinagre de Montilla-Moriles" PDO [1].

To obtain these protected vinegars, in addition to the acetous

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fermentation of their corresponding PDO wines made in the same production area, a special system of aging and maturation is necessary. This system is the classic "criaderas y solera" method, to which the vinegars are subjected during the period of time necessary to achieve the organoleptic and analytical qualities of their respective categories. Thus, within the "Vinagre de Jerez" PDO, the categories according to the different aging periods are: "Vinagre de Jerez" or commonly called "Crianza" (aged at least six months), the "Reserva" category (aged at least 2 years) and the less produced, but the most aged vinegar, "Gran Reserva" (with a minimum aging period of 10 years) [2]. "Vinagre de Montilla-Moriles" PDO includes the same categories above described [3]. Finally, "Vinagre de Condado de Huelva" PDO produces also different types of vinegars according to their aging: "Vinagre Condado de Huelva" that is a category not included in the other two PDOs, which includes wine vinegars not aged; "Solera" (aged for a period no less than six months), that is similar to the "Crianza" category in the aforementioned two PDOs, and "Reserva" (aged at least 2 years) [4].

PDO wine vinegars have higher prices in the market than other vinegars due to their raised quality and their costly production, in contrast with the rapid vinegars obtained by the submerged acetification, which are sold with lower prices due to their lower qualities. As result, adulteration and unfair competition in the vinegar industry are commonly practiced, leading to the need to characterize and differentiate the vinegars according to their quality. In this context, different analytical techniques have been applied for an extensive characterization and authentication of high-quality food products, allowing to protect their brands and to prevent adulteration and counterfeit [5–9].

These food quality control techniques are often based on instruments that involve quantification of compounds, usually taking long time and high costs, and also requiring well-trained analysts. However, some techniques that provide low time of analysis instead of a high accuracy, direct measurements and qualitative more than quantitative aspects, have currently demonstrated their ability to characterize wines vinegars by means of simple procedures [6,10–12]. Among them, rapid, non-destructive and direct methodologies based on non-targeted techniques, without the use of chemical references, are becoming more interesting for an authentication approach and determination of the geographical origin of food [13]. In this group, some spectroscopies, coupled with chemometrics, are nowadays one of the most applied techniques in food authentication [14–16].

In previous works, some spectroscopic techniques were tested in wine vinegars [10,11,17]. However, one of the simplest spectroscopic technique such as ultraviolet–visible (UV–vis) has not been studied yet for classification purposes of wine vinegars, although it has been successfully applied in many other food authenticity studies [18–21]. Moreover, a method using UV–vis spectroscopy applied to the classification of vinegars produced from different raw materials such as rice, mille, black rice, sticky rice, wheat bran, barley, sorghum, pea and mulberry, has been developed [21,22], which results support its possible ability of classifying PDO wine vinegars.

UV–vis spectroscopy is a technique based on measuring the absorption of UV and visible radiation by molecules, being the UV–vis region of the spectrum the wavelength that ranges from 190 nm to 800 nm [23]. The spectral position of an absorption band is indicative of the presence or absence of certain structural features or functional groups. The main reasons of interest in this methodology are its wide applicability, fastness of analysis, absence of generated residues and its ease of use, with no costs and no skilled operator. In addition, it has also a high sensitivity, moderate-high selectivity and good accuracy [15]. Thus, some authors have pointed out the possibility to discriminate one denomination of origin from others, in other food matrices, by using UV–vis spectroscopy and chemometric procedures such as principal component analysis (PCA) and soft independent modelling by class analogy (SIMCA) [20,24,25]. All of these characteristics make UV–vis an appropriate technique for controlling the productive processes, as well as monitoring and assessing composition and quality of products in food and beverages, pharmaceutical, and biological samples [26,27]. Furthermore, the possibility of developing new portable UV-spectroscopic devices for testing the authentication of some foods and beverages at field [28], increases the interest on this spectroscopy.

Most of the multi-class classification problems in food analysis focus on a small number of possible predictions. For example, a food product could be classified as proceeding from a determined origin, or from the specific raw materials used to produce it. However, in some occasions, both characteristics are wanted to know simultaneously, i.e. the specific origin where this food product is made and concurrently, which raw material was used for its production. In this last case, there are too many characteristics to consider at once, even some of them can be shared among both groups, which could be relevant for their classification or characterization. Actually, this complexity situation could be successfully solved by hierarchical models.

Hierarchical multi-label classification (HMC) is a variant of classification where an object may belong to multiple classes at the same time and these classes are organized in a hierarchy, as a tree of categories [29]. The organization in hierarchy means that an object that belongs to some class automatically belongs to all its super-classes [29]. Many important classification problems in the real-world need hierarchical classification systems, such as taxonomy, in which an object belongs successively to a specie, a genus, a family, and an order [30]. There are different types of hierarchical classification approaches. Thus, one is the so-called *flat classification approach*, which is the simplest one and consists on completely ignoring the class hierarchy, typically predicting only classes at the leaf nodes (i.e. tree-level). But the most used approach in the literature by far are the *local classifier per node approach* that consists on training one binary classifier for each node of the class hierarchy, and the *local classifier per parent node approach* that trains a multi-class classifier for each parent node in the class hierarchy to distinguish between its child nodes [30]. In spite of their advantages, not many studies have implemented this classification in terms of food classification [31,32].

Hierarchical classification approaches have some drawbacks. One problem is due to the fact that the HMC chains decisions and thus the error is propagated to each subsequent step. Other common problem to all types of classifications, but particularly important in the hierarchical case is that, as we go through the structure of the hierarchical model, the amount of data presented in each step is reduced as a consequence of focusing only on a subset of classes, and therefore, the reliability of the model could get worse. This problem could be solved by collecting a higher amount of samples of all the classes, but sometimes this is impossible. However, in this last case, some re-sampling algorithms could be applied to solve this problem. Re-sampling methods are widely used to estimate parameters and/or their uncertainty in a model, being the uncertainty estimation an important parameter to be evaluated in analytical data [33]. Bootstrapping is one resampling technique that is used in statistics more and more frequently. It was introduced by Efron [34] and it makes possible to solve difficult tasks when the size of samples is very small or when there are many classification levels in the structure, as obtaining confidence intervals, tests of statistical significance or any other statistics. The idea is to generate multiple sets of data that, after the analysis, shows the statistical variability of interest. For that, bootstrapping extracts successive samples from one real sample by re-sampling with replacement, so that some elements will not be selected and others may be selected more than once in each sampling to make new simulated samples. Therefore, bootstrap estimates standard errors from the empirical (original) data by re-sampling, allowing to calculate the confidence intervals for each sample as well as the reliability in the classification models by the knowledge of the uncertainty of each group defined in the hierarchical model [35].

In this context, the aim of this work was to develop a hierarchical classification model based on analytical data using a rapid, inexpensive and simple instrumentation such as UV–vis spectroscopy combined with chemometrics. Consequently, the purposed objectives were: a) to differentiate and classify the origin of the three Spanish PDO wine

vinegars, b) to discriminate their aging categories and c) to differentiate them from wine vinegars without a PDO. The reason of developing a classification approach at different levels is explained by the fact that each wine vinegar belongs to different classes according to what characteristic or quality parameter are considered. Thus, one vinegar can be aged or not aged, with or without a PDO, from different PDOs and for different aging categories. Thus, the proposed approach aims at being able to directly and easily make automated predictions on unknown wine vinegars without the need of quantification or trained specialists. Thereby, SIMCA and PLS-DA models were employed for this proposal with evaluation of the reliability of the results using the re-sampling bootstrap technique, which generate new data sets from the available one by an artificial perturbation.

2. Materials and methods

2.1. Wine vinegars

A total of 50 PDO wine vinegars of different categories within the 3 PDOs have been analyzed: 21 from the "Vinagre de Condado de Huelva" PDO (being 7 of the "Non-aged" category, 7 of the "Solera" category and 7 of the "Reserva" category); 16 from the "Vinagre de Jerez" PDO (being 7 of the "Crianza" category and 9 of the "Reserva" category); and 13 from the "Vinagre de Montilla-Moriles" PDO (6 and 7 of the "Crianza" and "Reserva" categories, respectively). All of them are "Aged" vinegars except the category "Non-aged" from "Vinagre de Condado de Huelva" PDO. All these PDO wine vinegars are made by a surface method of production. These PDO wine vinegars have been proportioned by the corresponding Regulatory Councils.

In addition, 20 wine vinegars without a PDO (so-called rapid vinegars) were purchased from the market and included in the study in order to authenticate a PDO from those without this designation. All these wine vinegars were made by a submerged method, being the reason for they were named "rapid vinegars". Among these vinegars, Spanish and Argentinian wine vinegars were included (7 and 13 samples, respectively). Although the majority of them are "Non-aged" (all of the Spanish origin), some of the Argentinian vinegars (that are still not registered as a PDO) presented some time in wood barrels, so they were included as "Aged" vinegars but without a PDO. More information is shown in Table 1.

2.2. Sample preparation

As the direct analysis of the wine vinegars produced signal saturation in the detector of the spectrophotometer, a previous dilution study was necessary. Thus, prior to the analytical determinations by UV–vis, in order to optimize the spectral conditions of analysis and to acquire suitable spectroscopic information about vinegar samples, different dilutions with ultrapure water were tested. The study of the optimal dilution was carried out from the following vinegar/water (v/v) ratios: 1/20, 1/10, 1.5/10, 2/10 and 4/10 and without dilution. According to the results, it was decided to work with the dilution 1/10 vinegar/water (v/v), as a commitment situation due to the differences of intensities presented through the samples, because this dilution allowed to record the spectrum of highest intensity samples, but also allowed the observation of the spectral curves from those with the lowest signal. As consequence, this dilution did not saturate the spectra and did not lose the signal of the least dark samples, and therefore, being adequate for all classes.

2.3. UV–vis analysis

UV–vis spectroscopy measurements were performed using a spectrophotometer UV–vis Ocean Optics CHEMUSB4 coupled with a detector with diode array. The samples were placed in a quartz cuvette with a path length of 10 mm. The absorbance as a function of wavelength was measured with a resolution of 2 nm in a working range from 180 to 890 nm by duplicate. Ultra-pure water (MilliQ quality) was used as the reference scan.

2.4. Data analysis and software

Once the spectra were collected, in order to remove noise, a specific wavelength range was selected (from 280 to 600 nm) as the informative region (Fig. 1). Different preprocessing methods were evaluated, and the best one in terms of the explained variance obtained in the models was the standard normal variate (SNV) method. Then, the data was mean centered prior to analysis.

Firstly, Principal component analysis (PCA) was employed as exploratory analysis for wine vinegar samples. Similarities and differences between samples were studied by observing the scores plots, and the weight of variables were studied by the loadings plots. Several PCA models were obtained according to the different classes studied (i.e. "Aged", "Non-aged", PDO, Non-PDO, etc.).

Secondly, a hierarchical classification model (HCM) was developed by using soft independent modelling by class analogy (SIMCA) and partial least squares-discriminant analysis (PLS-DA) according to the different classes studied. SIMCA is a supervised classification method in which samples belonging to each class need to be analyzed using PCA and retaining the significant principal components. PLS-DA is another supervised classification method based on searching the optimal latent variables for discriminating between the classes [36].

Prior to the classification models, samples were randomly divided into two data sets: training and test set -with 75 and 25% of samples, respectively-including all categories, and using Venetian blinds cross-validation in each step of the classification approach. In order to validate the models and to evaluate the reliability of the results, the non-parametric bootstrap re-sampling method was applied to estimate the uncertainty and to obtain the confidence limits of the model. The bootstrap employed in this study was extracted from Babamoradi, Van den Berg & Rinnan, (2013) [35].

Briefly, the non-parametric bootstrap method consists on re-sampling by a random selection of samples, not needing the assumption on the uncertainties distribution due to it is estimated by the bootstrapping procedure [35]. Moreover, bootstrap provides reliable and consistent confidence limits (CLs), which are the most common way to estimate the uncertainty. In this study, bias-corrected accelerated method (BCa) was used to build the CLs [35,37,38]. With the bootstrap distribution, the central value (the estimated point) and the confidence intervals (confidence limits) can be calculated in a similar way as the confidence interval of a mean from the sampling distribution. Thus, bootstrap was applied in

Table 1
Samples included in the study.

Class		Aged vinegars						Non-aged vinegars		
Time of aging		Aged >6 months	Code	N	Aged >2 years	Code	N	0 months	Code	N
PDO	"Vinagre de Condado de Huelva"	"Solera"	CSO	7	"Reserva"	CRE	7	"non-aged"	CSC	7
	"Vinagre de Jerez"	"Crianza"	JCR	7	"Reserva"	JRE	9			
	"Vinagre de Montilla-Moriles"	"Crianza"	MCR	6	"Reserva"	MRE	7			
Non-PDO	Spanish "rapid vinegars"	–						RV		7
	Argentinian "rapid vinegars"	RV				3		RV		10

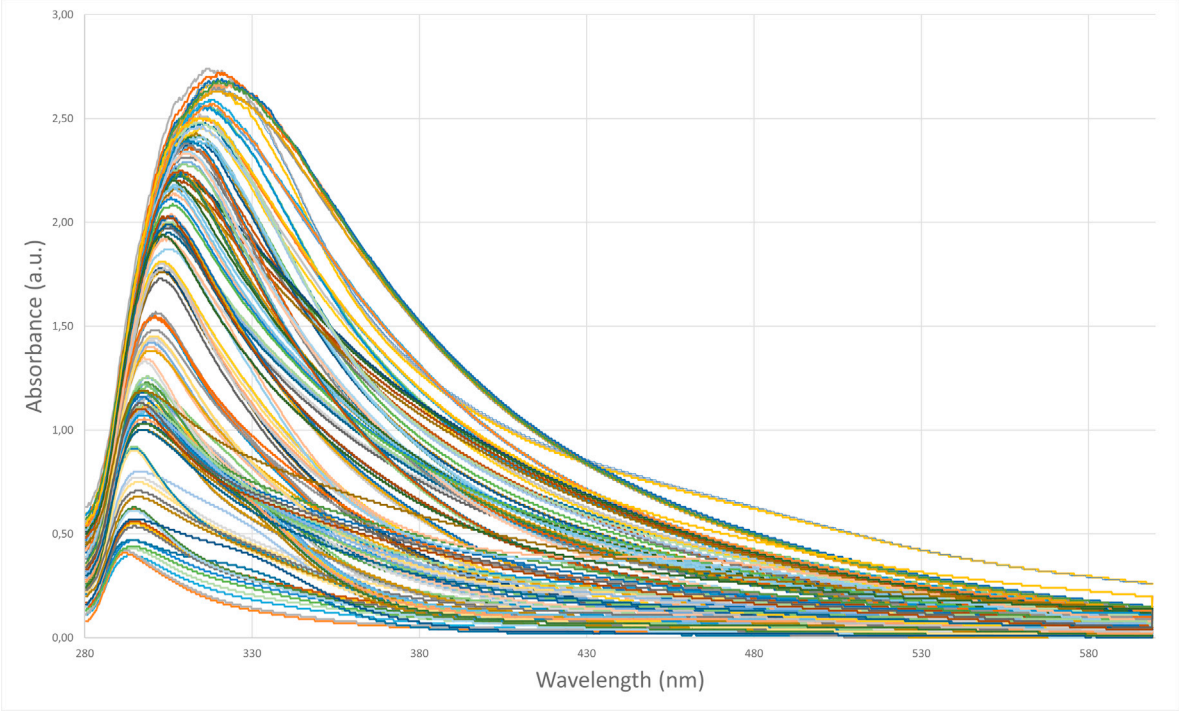

Fig. 1. Raw absorbance spectra of the samples from 280 to 600 nm spectral region.

the last step of the hierarchical tree, that is, the SIMCA classification within each PDO according to the aged category, where the amount of samples for each class was small. The number of samples *N* in the train set for each PDO was multiply by 40 by bootstrap, so that, the calculation of bootstrap was performed as follows: 12 × 40 re-sampling for CSO and CRE, respectively; 12 × 40 and 14 × 40 for JCR and JRE, respectively; and 10 × 40 and 12 × 40 for MCR and MRE, respectively. The information about samples and codes are shown in Table 1. Bootstrap BCa CLs (at 95% of confidence level) were built from PCA model parameters that conforms the SIMCA models. The same number of PCs as each SIMCA model were used. Thus, for the set of samples from "Vinagre de Condado de Huelva" PDO, 3 and 2 PCs were used to build the PCA model of CSO and CRE, respectively; for "Vinagre de Jerez" PDO set, 4 PCs were used to build both PCA models of JCR and JRE; and for "Vinagre de Montilla-Moriles" PDO, 2 PCs were used to build the PCA model for MCR category and 3 PCs for the PCA model of MRE samples. Moreover, as for all the classification models, spectra were pre-processed by SNV and data were mean-centered prior to bootstrap.

All data analysis was performed using the PLS_Toolbox 7.9.5 working under MATLAB environment version 2017a.

3. Results and discussion

3.1. Visualization and exploration of the spectra

3.1.1. "Aged" vs "Non-aged" vinegars

The first criterion used to differentiate the PDO wine vinegars under study was according to whether they were or not aged, due to, the major part of wine vinegars with PDO are aged, according to their legislation [1], whereas the wine vinegars without aging were mainly Non-PDO. However, there were a few exceptions such as the category without aging that belongs to the "Vinagre de Condado de Huelva" PDO and some wine vinegars that were produced in Argentina by a traditional method and were aged for a period of time, but they do not have a PDO indication.

First, a visual assessment of the UV–vis spectra for each group, "Aged" and "Non-aged", was carried out. As it could be observed in top of Fig. 2 (Fig. 2A), which shows at the left side the raw spectra of the wine vinegars according to the groups "Aged" and "Non-aged", some differences could be highlighted. First of all, it could be observed, in general, a difference in the intensity of the absorption bands between both groups. "Aged" wine vinegars showed a higher intensity than "Non-aged" wine vinegars. However, the intensity of the bands was not only the main difference between samples, because the shape of the spectra also had a high relevance in the difference of both groups, mainly in the differentiation between PDO and non-PDO wine vinegars.

Then, in order to better study the differentiation between these groups, a PCA model was developed including all the samples under study and grouped in "Aged" and "Non-aged" classes. Fig. 2A shows the score plots and the 95% confidence ellipse obtained by the PCA model carried out with these samples. The scores plot of the first and the four principal components (PCs), that explained 86.29% of total variance, showed a trend of grouping the "Aged" and "Non-aged" samples. Thus, wine vinegars without aging (i.e. "Non-aged") were placed in the positive side of PC1, whereas the "Aged" wine vinegars were in the negative side of PC1. It could be also observed some overlapping between samples that occur due to the differences presented according to the origin, PDO or category taken into account. However, these samples were separated by the other PCs.

The loadings plot of PC1 showed that the main differences between the "Aged" and "Non-Aged" wine vinegars were explained by an intense band of the spectra around 300 nm for the "Non-aged" wine vinegars, and the spectral range between 325 and 450 nm that mainly explained the "Aged" wine vinegars. This last one was the region where some differences in the shape were observed in the original spectra. During aging, some changes occurs in the vinegar, such as evaporation of water and therefore concentration of compounds, the transference of components from wood to vinegar and oxidation reactions. Therefore, the higher or lower presence of some compounds could be related to the difference in the absorbance bands. According to the literature, many of these compounds that increase with oak-aging time in wines and vinegars can be furfurals, whiskey lactones, syringaldehyde, as well as other phenolic and volatile compounds [39].

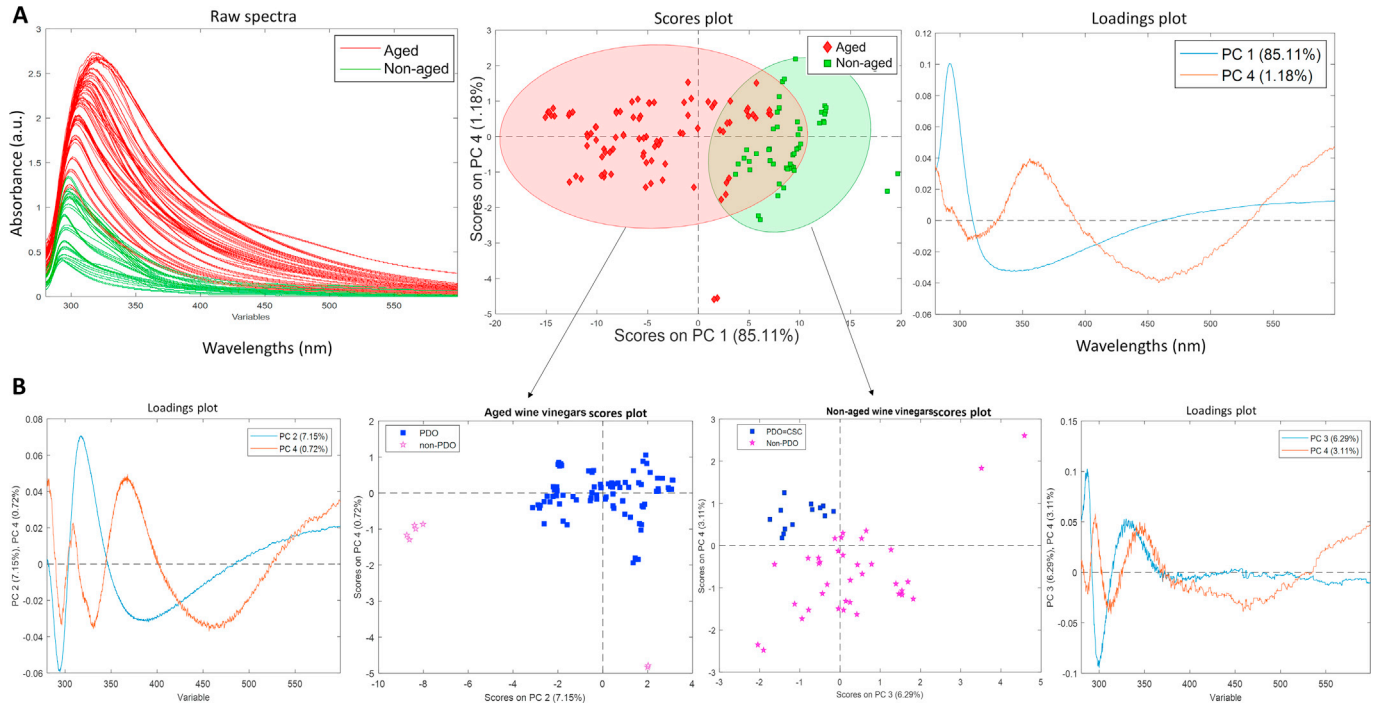

Fig. 2. Raw spectra, scores and loadings plots about: A) the PCA model developed by grouping and colouring samples into "Aged" and "Non-Aged" wine vinegars; B) the PCA models developed for the group of "Aged" and the group of "Non-Aged" separately, grouping and colouring samples into PDO and Non-PDO wine classes.

3.1.2. *PDO vs Non-PDO*

In order to assess the ability of the method in the separation of wine vinegars with and without a PDO, each group -evaluated as "Aged" or "Non-aged"- was studied separately. Fig. 2B shows the scores and loadings plot for two developed PCA models, one for each group according to aging. In both cases, "Aged" and "Non-aged", the separation between PDO and Non-PDO samples was observed. The number of samples of "Non-PDO-Aged" vinegars was small because, as was mentioned above, these samples were those produced in Argentina with aging but without a PDO. A similar case occurred with "Non-Aged" wine vinegars with PDO, due this group was formed by the "without aging" category of "Vinagre de Condado de Huelva" PDO.

By looking at the loadings plot in the right and left side of Fig. 2B, once again the absorption bands between 300 and 450 nm seemed to be key spectral ranges for the separation of groups, although in this case other spectral regions were involved. Thus, in the case of "Aged" vinegars, those without a PDO were explained mainly by negative loadings for PC2 related to the band at 290 nm and 400 nm. By contrary, "Non-aged" wine vinegars with PDO (i.e. CSC) were separated from the others and placed in the negative side of PC3 and positive side of PC4, being associated to a spectral band at 300 nm.

Several compounds absorb in these relevant regions of the spectrum and discrimination was possible due to the different concentration of them according to the type of sample. Thus, aromatic compounds that have an important effect on the quality of vinegars, have also showed differences between high-quality vinegars from the others [40–42]. Thus, Theobald et al. (1998) demonstrated that the concentration of 5-hydroxymethylfurfural, a product that comes from sugar and storage time, was higher in sherry and balsamic vinegars than in white and red wine vinegars without a PDO [43]. Moreover, the phenolic composition has been demonstrated to be useful for determining the method by which vinegar is produced, as well as for determining different periods of aging. For this reason, phenolic compounds could be valuable to differentiate wine vinegars with PDO obtained by traditional long system from those made by quick acetification as the "rapid vinegars" [44,45]. That is also the case of some aldehydes such as benzaldehyde, syringaldehyde, and vanillin, which have been more frequently found in vinegars elaborated by slow traditional methods than in quick vinegars [44]. Furthermore, certain compounds are also chemical markers of the wood in which the vinegar has been in contact with [39]. Accordingly, high-quality vinegars contain a large number of these compounds at high concentrations, reason why this characteristic has been selected as attribute of high quality vinegars [46]. Other studies that have assessed vinegars with or without PDO, have also demonstrated that sensory differences between products made by traditional methods in which vinegar is aged in wood barrels, from those manufactured at industrial scale also exist [47]. The observed UV–vis spectra, as well as the results observed in scores and loadings plots of PCA, could reaffirm that the PDO wine vinegars had unique quality and characteristics that could allow their differentiation from non-PDO, as well as aged vinegars from the rapid and non-aged ones. Moreover, as the non-PDO wine vinegars are obtained by submerged method of production and the PDO wine vinegars are obtained by a surface method, it could be assumed that UV–vis could also be able to differentiate both production methods.

3.1.3. *Differences between PDOs within the same category*

The next step was to differentiate samples of the same category but different PDO. Thus, PCA models were developed only including PDO samples in order to study this differentiation. Fig. 3 shows the raw spectra of the PDO samples with different categories (Fig. 3. **A1,B1,C1**) and the scores (with confidence intervals of 95%) and loadings plots of the PCA models developed for each category (Fig. 3. **A2-3, B2-3, C2-3**).

By simple observation of the spectra (Fig. 3. **A1, B1 and C1**), it could be easily seen a difference in the intensity in the samples of each PDO independently of the category of aging. Thus, samples belonging to "Vinagre de Jerez" PDO (JCR and JRE) showed the highest intensity (around 2.5 a.u.) showing a maximum around 330–360 nm, followed by aged samples from "Vinagre de Condado de Huelva" (CSO and CRE), with an intermedium intensity and a maximum peak around 330 nm, "Vinagre de Montilla-Moriles" (MCR and MRE) samples showing less intensity and a maximum peak around 295–310 nm, and finally the "Non-aged" "Vinagre de Condado de Huelva" vinegars with the lowest intensity (around 1 a.u.) and a maximum around 290 nm. There could be also appreciated a slightly difference in the shape of the spectra, mainly

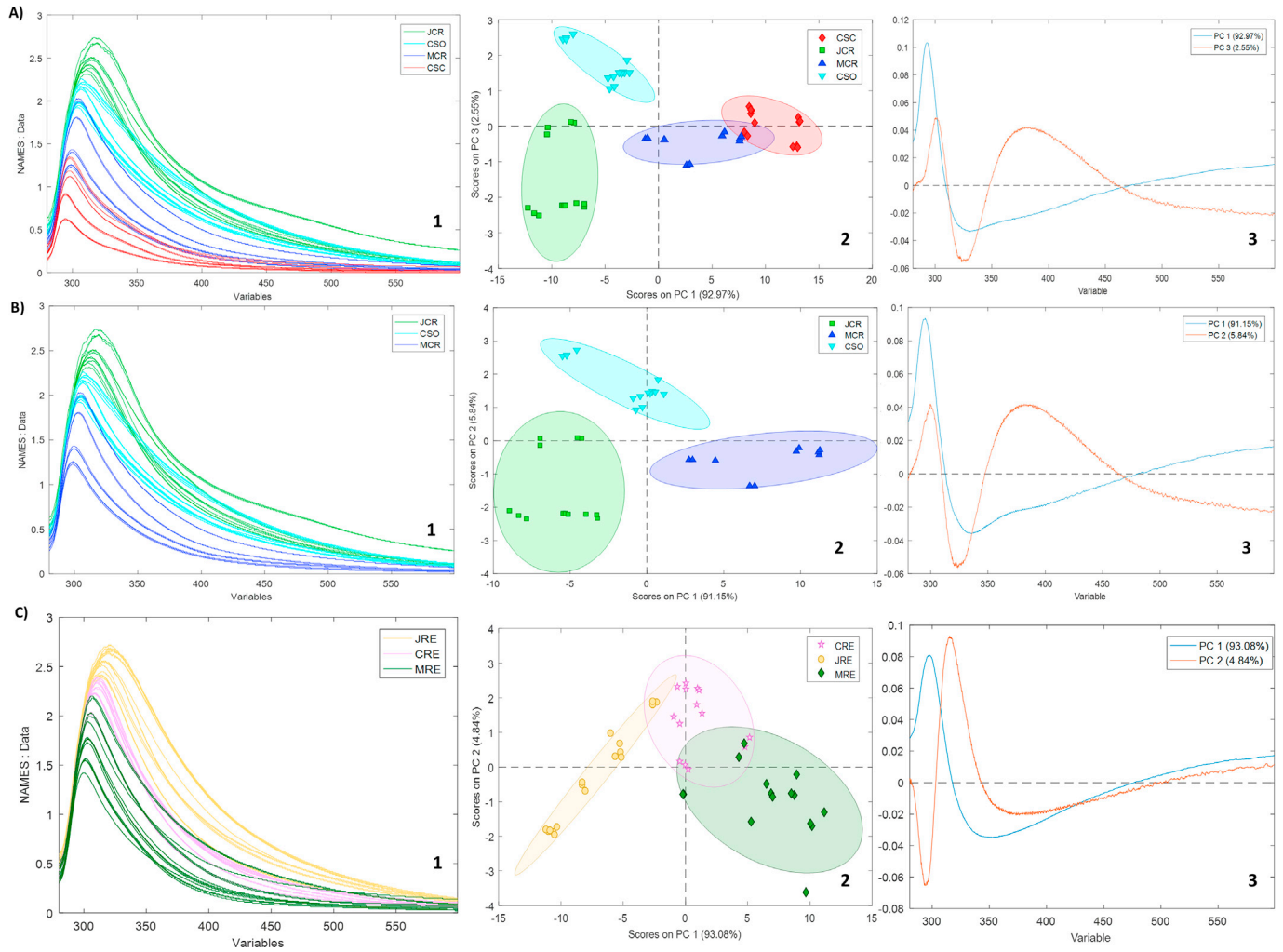

Fig. 3. Spectral profile (1) and the score and loadings plot of a PCA model (2 and 3, respectively) developed with the least aged categories (CR and SO) and the Non-aged category (CSC) of the three PDOs (**A**). Spectral profile (1) and the score and loadings plot of a PCA model (2 and 3, respectively) developed with only the least aged categories of the three PDOs (CR and SO) (**B**). Spectral profile (1) and the score and loadings plot of a PCA model (2 and 3, respectively) developed with the "Reserva" category of the three PDOs (**C**). The acronyms for the different vinegar categories are defined in Table 1.

for CSC category.

A PCA model was developed with the least aged PDO wine vinegars (Fig. 3A): "Crianza" for "Vinagre de Montilla-Moriles" and "Vinagre de Jerez" PDOs (coded as MCR and JCR, respectively) and "Solera" (CSO) from "Vinagre de Condado de Huelva" PDO. Moreover, vinegars without aging (CSC) for "Vinagre de Condado de Huelva" PDO were included in the model. Thus, PC1 explains 92.97% of the total variance and clearly showed a separation between JCR and CSO, in the negative side of PC1 (Fig. 3. **A.2**), from MCR and CSC in the positive PC1 side, having this last category more positive PC1 scores than MCR. Moreover, JCR and CSO were separated by PC3. The similarity between MCR and CSC could explain that MCR samples were aged for the minimum period of aging allowed for this category (i.e. 6 months), whereas JCR and CSO categories could be aged for more than 6 months.

A second PCA model was developed by including the least aged vinegars but in this case, without the "Vinagre de Condado de Huelva" and "Non-aged" category (CSC), in order to study the most similar wine vinegars from the three PDO (Fig. 3. **B2-3**). The included samples belonged to a category legislated with a minimum period of 6 months and a maximum of 2 years of aging in wood barrels (named as "Crianza" and "Solera" categories), so in this case, all of them should have some effects of being stored in wood for a period of time and the differences might be more related to the origin. Once again, the separation between PDOs was clearly observed: MCR was placed in the positive side of PC1, CSO in the positive side of PC2, and JCR in the negative side of PC1 and PC2. By looking the loadings plots of these PCA models (Fig. 3. **A3 and B3**), the main wavelengths responsible for the distinction of PDOs were found around 295 nm and from 500 to 600 nm for MCR samples, around 325–350 nm for JCR samples and around 350–450 nm for CSO samples. These results matched with those observed by the direct visualization of the spectral profile.

The "Reserva" category of each PDO was also studied by separate (Fig. 3C). These samples were similar between them, due to they had to be aged for at least 2 years in wood barrels, independently of the PDO. Regarding the scores plot (Fig. 3.C2), a good separation was achieved according to the first two PCs. PC1, which accounts for 93.08% of total variance, seemed to be responsible of the separation of the JRE samples, with negative values, from MRE samples. In the same way, MRE samples were separated from CRE samples mainly by PC2. The loadings plot (Fig. 3.C3) showed again the importance of the spectral regions between 300 and 350 nm above mentioned.

As it is known, UV–Vis spectrum represents the information based in the composition of absorbent species, such as phenolic, benzoic, and hydroxycinnamic acids, polyphenolic compounds, and also stilbenes, flavanols, and anthocyanins. Some of these compounds absorb in the ultraviolet region between 300 and 400 nm, which has shown to be

mainly responsible of the differentiation of PDOs, and also they have been related to the color and taste characteristics of wine and wine vinegars [20]. Among them, phenolic compounds of wine vinegars had shown to be useful for differentiating them according to the elaboration method applied or the geographical origin of the wine substrate. Thus, according to García-Parrilla et al. (1997), compounds such as (hydroxymethyl)-furaldehyde, tyrosol, vanillic acid, vanillin, and caffeic acid were selected as the relevant for a classification according to geographical origin [45]. Moreover, Urbano et al. (2006) also observed that some esters and hydroxycinnamic acids absorb at these ultraviolet regions and were responsible of the discrimination of wines by their origin [25]. Therefore, from the present exploratory analysis, it can be seen that the origins are clearly separated, probably due to differences in composition of these aforementioned compounds in each geographical origin. The other conclusion of this exploratory analysis that can be extracted by observing the spectral regions where the differences occur is that the discrimination could not be visual, i.e. related only to the color, since the differences mainly lead in the ultraviolet region. This fact was also observed by other authors that used UV–vis for the discrimination of wines [20,25].

3.1.4. *Differences between categories of each individual PDO*

Once differences between PDOs have been analyzed, each PDO was assessed separately in order to study the differences between categories within each PDO. Thus, the different categories produced and commercialized for each PDO according to aging in wood barrels were analyzed by the development of new PCA models. The raw spectra and the scores and loading plots of the three PCA models developed, one for each PDO, are shown in Fig. 4. It should be highlighted that in this case, the reduction of variables was very important to achieve a good separation of categories. As it can be observed in the plot of the raw spectral profiles (left side of Fig. 4), the differentiation between categories for each PDO was not based on the intensity of the signal, as occured with the PDO-discrimination, but it was based in the overall shape of the profile.

By looking at the score plots, a good separation of categories could be observed in the three models, although the best separation was obtained in the PCA model of "Vinagre de Condado de Huelva" PDO (Fig. 4B). In this model, the three categories were placed completely separated in different quadrants of the scores plot. The scores plot for samples of "Vinagre de Montilla-Moriles" PDO showed also a good separation of categories. However, some overlapping appeared in the PCA model of "Vinagre de Jerez" PDO (Fig. 4A). Thus, in spite of the "Crianza" and "Reserva" samples were placed separately mainly by means of PC3, this was not perfectly clear for some samples, also having different groupings within each category. These differences could be explained by the fact that the different samples of each category from "Vinagre de Condado de Huelva" PDO were from the same wineries, so the differences between them may be only due to the effect of aging. However, in the other two PDO, due to they belonged to different wineries, the aging effect was also affected for differences in the elaboration.

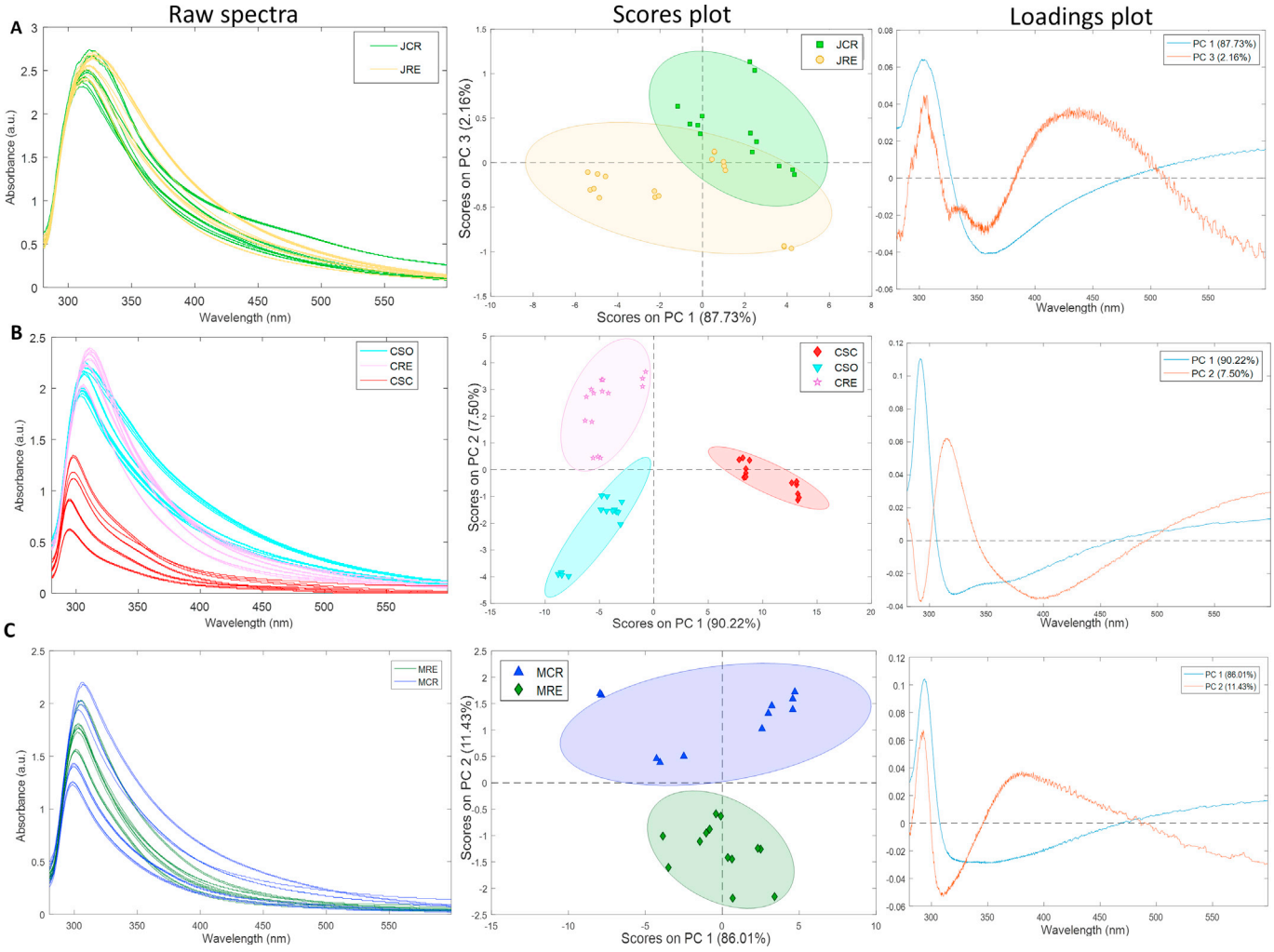

Fig. 4. Score and loading plots of the three PCA models developed, one for each PDO, showing the different aging categories within each PDO. The acronyms for the different vinegar categories are defined in Table 1.

According to the loading plots (right side of Fig. 4), some similarities and differences between the three models could be observed. Thus, the spectral region around 300 nm and the visible range from 500 to 600 nm seemed to be, in general, responsible of the "Reserva" samples, whereas the least aged categories (Crianza and Solera) for each PDO were more related to absorption regions around 290 nm and between 350 and 500 nm. However, some differences could be also noticed for these categories within each PDO. In "Vinagre de Jerez" PCA model, the JRE category was explained by the above mentioned regions, but showed the maximum absorption around 330 nm with a shoulder around 360 nm (with the most negative values of PC3), while the JCR samples were mainly explained by a peak at 310 nm and the region between 400 and 500 nm. Regarding the PCA model of "Vinagre de Condado de Huelva" PDO, the loadings showed a maximum peak in the positive loadings of PC2 at 330 nm that explained the CRE samples, while the CSO samples were explained by the region between 350 and 450 nm while the "Non-aged" PDO category (CSC) by a high positive PC1 loadings around 290 nm. Finally, the loadings plot obtained for the model of "Vinagre de Montilla-Moriles" PDO showed that the spectral region more related to MRE samples -corresponding to those with negative values of PC2- was placed in the region closer to 300 nm, while for MCR samples the related regions were around 290 nm and between 350 and 450 nm.

According to the literature, the group of polyphenols is one of the groups of compounds related to the aging, i.e. to the "Rerserva" category, which can be seen by UV–vis in the above mentioned spectral ranges. The phenolic composition has proved to be useful to determine the method by which vinegar is produced (submerged or surface acetification), as well as for determining the different aging periods [48]. Thus, for example, within this group of compounds, authors as García-Parrilla et al. (1999) and Tesfaye et al. (2002) showed that gallic acid, vainillic acid or hydroxymethylfuraldehyde were presented in higher concentrations in very aged vinegars than in less aged vinegars of the PDO "Vinagre de Jerez" [49–50]. Other absorbent species that have shown to be present in aged wines and vinegars and are able to be represented in the UV–vis spectra are phenolic, benzoic, and hydroxycinnamic acids and stilbenes, flavanols, and anthocyanins [20]. Moreover, the relevance of the visible region of the spectrum (500–600 nm) in the "Reserva" samples could be explained by the change of colour that occurs during aging, because storaging in wood barrels produces changes in the darkening of colour in comparison to the less aged vinegars [49].

3.2. Hierarchical classification by SIMCA and PLS-DA analysis

A hierarchical classification model was developed (Fig. 5) by using soft independent modelling by class analogy (SIMCA) and partial least squares-discriminant analysis (PLS-DA) in the different nodes. The classification results of the different models (assessed by sensitivity, specificity and percentage of correct classification of the different models) are shown in Table 2.

The first classification level (named as root) consisted on classifying the training samples into two general classes, "Aged" and "Non-Aged". A SIMCA model (numbered as SIMCA 1 in Fig. 5 and Table 2) was carried

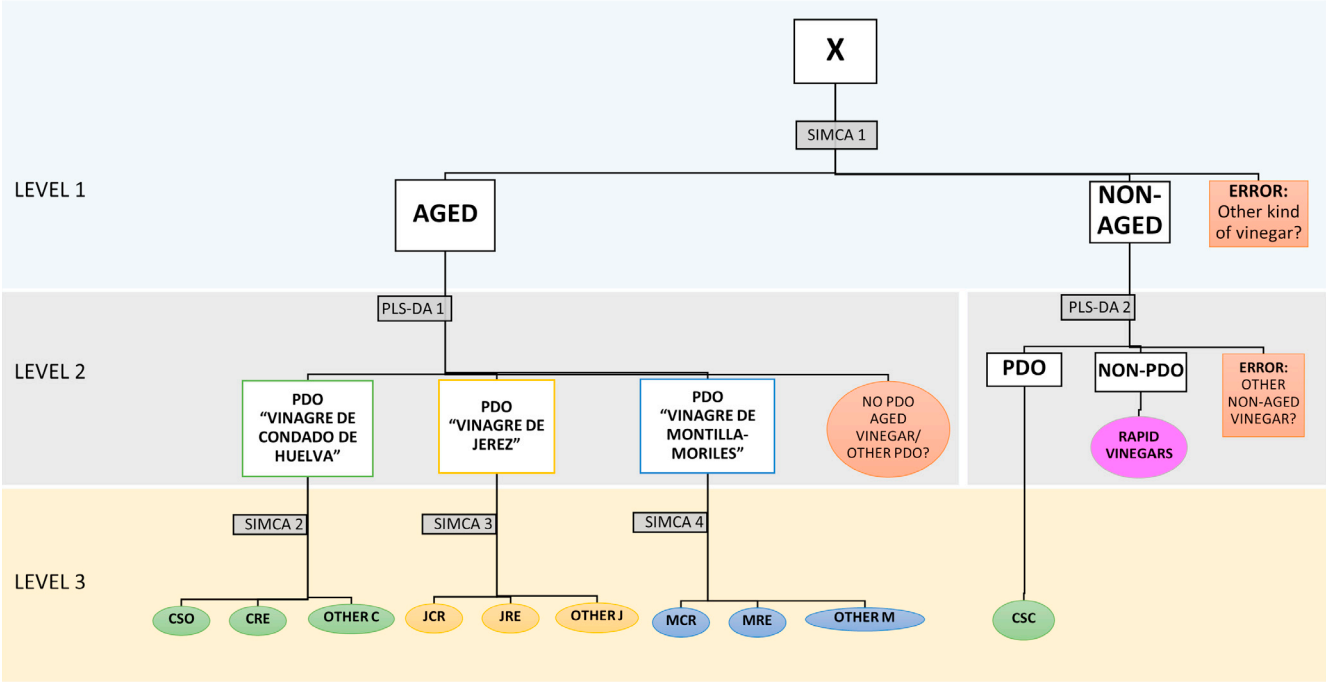

Fig. 5. Flowchart of the hierarchical classification model developed. The acronyms for the different vinegar categories are defined in Table 1.

Table 2
Classification results of the SIMCA and PLS-DA models used for the construction of the hierarchical classification model expressed by the percentage of sensitivity, specificity and correct classification.

Model	SIMCA 1		PLS-DA 1			PLS-DA 2		SIMCA 2		SIMCA 3		SIMCA 4	
PCs or LVs	5	4	5			3		3	2	4	4	2	3
Class	AGED	NON- AGED	C	J	M	CSC	RV	CSO	CRE	JCR	JRE	MCR	MRE
Sensitivity CAL	91.3	100	96.0	92.0	91.3	100	100	85.7	100	100	93.3	100	100
Specificity CAL	100	91.3	77.2	95.8	94.0	100	100	100	85.7	93.3	100	100	100
% correct classification CAL	90.4	97.0	80.0	92.0	87.0	100	100	83.3	100	100	92.9	100	100
% correct classification PRED	100	100	100	100	100	100	100	100	100	100	100	100	100

out for that purpose by using a PCA model with five PCs for "Aged" samples and PCA model of four PCs for the "Non-aged" group. All the "Aged" samples were correctly classified, although three of them (i.e. MCR samples) were assigned to both classes. Moreover, only one of the replicates of a "Non-aged" sample was unassigned to any class.

The next level of classification was performed by PLS-DA in order to classify samples of the previous two groups into wine vinegars with and without a PDO. Regarding the "Aged" group, a PLS-DA model (numbered as PLS-DA 1 in Fig. 5 and Table 2) was built in order to classify PDO Aged samples according to the three Spanish PDOs ("Vinagre de Jerez", "Vinagre de Montilla-Moriles" and "Vinagre de Condado de Huelva"), together with another group in which "Aged" samples without a PDO were classified. AS it was explained before, this last class was formed by a few "rapid vinegars" made in Argentina, without a PDO, but aged for a short period of time. The best classification results were achieved by a five latent variables (LVs) PLS-DA model accounting a 99.71% of explained variance. The 80%, 92% and 87% of samples were correctly classified as "Vinagre de Condado de Huelva", "Vinagre de Jerez" and "Vinagre de Montilla-Moriles" PDOs, respectively, in calibration and cross-validation.

Within the "Non-aged" group, another PLS-DA model (numbered as PLS-DA2 in Fig. 5 and Table 2) was carried out by using three latent variables (LVs) and the 100% of the samples were correctly classified as PDO and Non-PDO in calibration and cross-validation. Samples classified as "Non-aged" and PDO wine vinegars were those wine vinegars belonging to the category CSC of "Vinagre de Condado de Huelva" PDO, due to these samples were not aged in barrels but they were made by the procedure described in the regulations of this PDO.

Among each PDO, the corresponding aged categories were classified by SIMCA in the last level of the hierarchical model (numbered as SIMCA 2, 3 and 4 in Fig. 5 and Table 2). Thus, for "Vinagre de Condado de Huelva" PDO, 3-PCs and 2-PCs PCA models were developed for CSO and CRE categories, respectively. All the samples were correctly classified except one CSO sample that was categorized as multiple class. Regarding "Vinagre de Jerez" SIMCA model, 4-PCs PCA models were developed for each category and all the samples were correct classified except one replicate of a JRE sample that was classified in both categories. Finally, for "Vinagre de Montilla-Moriles" PDO, SIMCA model was built by a 2-PCs PCA model for MCR category and a 3-PCs PCA model for the aged category MRE. A 100% of correct classification was achieved for this PDO.

Once the hierarchical model was made, the test-set with the samples of unknown class or category was used for validation of the model. The percentage of correct classification obtained showed that all the test samples were classified according to their labeled class or category (Table 2), which means that the hierarchical model provided a 100% of correct classification. Moreover, the probability (%) that each sample belongs to one category or class (Table 3) also revealed the good classification ability of the hierarchical model.

3.3. Validation of the hierarchical model by bootstrapping

One of the problems of a hierarchical model is that the number of samples in the last nodes or steps are reduced as a direct consequence of focusing only on a subset of outcomes. Moreover, the aforementioned classification results by means of the figures of merit of the models (i.e. sensitivity, specificity …) give an overall vision of the behavior of the model, but do not give information about individual samples (i.e. the error for each sample is not computed). For these reasons, in addition to the cross-validation procedure carried out in each model, the bootstrap re-sampling method was performed for the last step of the hierarchical tree where the amount of sample was reduced, that is, in the SIMCA classification within each PDO according to the aged category (SIMCA 2, 3 and 4 in Fig. 5).

Fig. 6 shows the bootstrap based on 95% BCa CLs of score values for the different PCA models, developed for each PDO category within each SIMCA model: "Vinagre de Condado de Huelva" PDO (Fig. 6A-B), "Vinagre de Jerez" PDO (Fig. 6C-D) and "Vinagre de Montilla-Moriles" PDO (Fig. 6E-F). Each score plot (Fig. 6 A, C and E) shows the bootstrap based 95% score values of one category and the predicted scores of the other one from which it wanted to be separated calculated by using the loadings values obtained for the first one. The calculated scores for the test samples of each category are also shown. The uncertainty estimation for samples was shown by the error bar in the scores plots of Fig. 6 **(A, C and E).** Moreover, the lower and upper 95% BCa CLs of each sample by the PCs mainly involved in the separation were also shown in the right part of Fig. 6 **(B, D and F).**

It could be seen that the CLs for the first principal component were nearly symmetric and of equal size for all objects of the same category. That means that, theoretically, the samples for this category may have similar spectral characteristics. Moreover, as it could be seen in each PCA score plot, all the samples were clearly separated by categories, even taking into account the confidence limits, which could confirm the reliability of the SIMCA model to classify between categories within a PDO. In addition, the prediction of the test samples for each model showed the correctly separation by categories of the different samples. These results show the reliability of the model to discriminate between categories within each PDO in spite of the low amount of samples for each one.

4. Conclusions

The present study showed for the first time the application of UV–vis spectroscopy for the authentification and differentiation of wine vinegars. In addition, this methodology was combined with the development

Table 3
Supplementary material. Mean values of probability expressed as % obtained for the each sample of the test set.

MODEL	Category									
	%probability	JCR	JRE	MCR	MRE	CSO	CRE	CSC	RV NOT AGED	RV AGED
SIMCA1	**%probability of being Aged**	100	100	100	100	100	100	0.0	0.0	80.0
	PDO	100	100	100	100	100	100	0.0	0.0	5.0
	NON-PDO	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	85.0
SIMCA1	**%probability of being Non-aged**	0.0	0.0	0.0	0.0	0.0	0.0	100	99.9	0.0
	BEING CSC	0.0	0.0	0.0	0.0	0.0	0.0	100	0.0	0.0
PLS-DA2	**BEING RV**	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100	8.0
PLS-DA1	**%probability of being C**	1.3	3.7	40.0	3.5	90.1	47.0	–	–	–
SIMCA2	**BEING CSO**	–	–	–	–	98.0	0.0	–	–	–
	BEING CRE	–	–	–	–	4.0	100	–	–	–
PLS-DA1	**%probability of being J**	99.9	99.5	0.02	0.06	0.2	18.0	–	–	–
SIMCA3	**BEING JCR**	60.4	3.5	–	–	–	–	–	–	–
	BEING JRE	2.68	58.0	–	–	–	–	–	–	–
PLS-DA1	**%probability of being M**	0.3	0.4	99.5	99.9	7.3	8.1	–	–	–
SIMCA4	**BEING MCR**	–	–	100	0.0	–	–	–	–	–
	BEING MRE	–	–	0.0	100	–	–	–	–	–

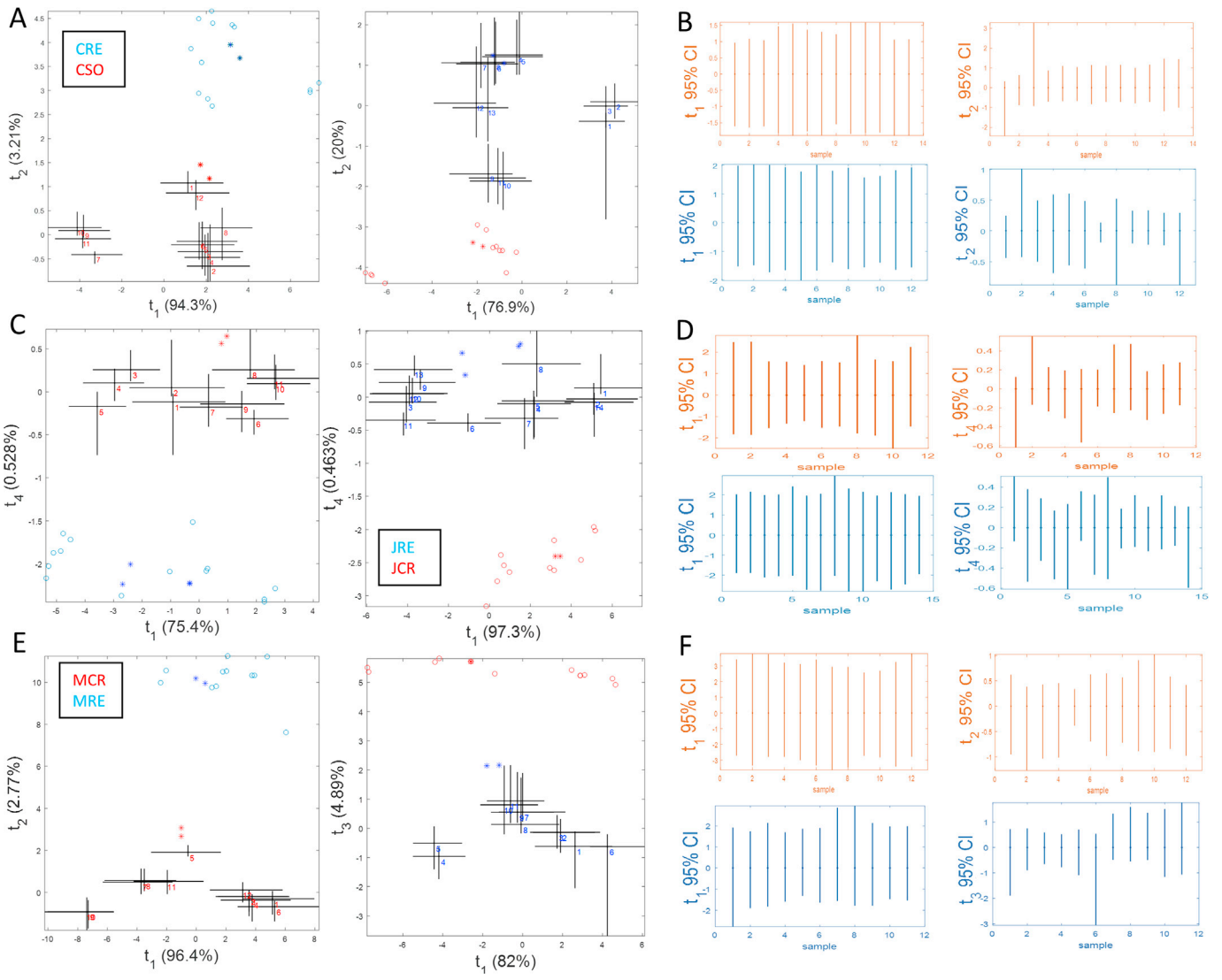

Fig. 6. Bootstrap based 95% BCa CLs for score-values of the different PCA models developed for each PDO category within each SIMCA model, with the uncertainty estimation for each sample plotted by error bars (**A, C and E**). Train samples are represented with 'o' and Test samples with '*'. Lower and upper 95% BCa CLs of each sample by the PCs involved in the separation (**B, D and F**). The acronyms for the different vinegar categories are defined in Table 1.

of an hierarchical classification model, by using PLS-DA and SIMCA methods and bootstrapping for the assessment of the uncertainty, with the aim of distinguishing between "Aged" and "Non-aged" wine vinegars, between PDO and non-PDO or rapid vinegars, between different PDOs and even between different categories of aging within a PDO. Moreover, it is the first time that all the classes are classified at once with only one analytical technique and by this chemometric methodologies.

The results obtained showed that the region of the UV spectra around 300 nm, and the visible region between 500 and 600 nm explained the differentiation of the most aged vinegars ("Reserva" category), within which in turn, each PDO showed a difference in the intensity and a displacement of these UV regions (i.e. JRE around 360 nm and the highest intensity, CRE around 330 nm and MRE closer to 300 nm an the lowest intensity). Moreover, the least aged PDO vinegars ("Crianza"and "Solera" categories) were better explained by the spectral range around 290 nm and between 350 and 500 nm. Thus, in general, JCR is more explained by the absorbtion bands at 310 and 330 nm aprox., MCR around 290 and 295 nm, and CSO at 290 nm plus the region around 400 nm. Finally, the Non-aged PDO vinegars (CSC) showed the lowest intensity and the principal peak at 290 nm.

Even though the information contained in the UV–Vis spectra is unspecific because compounds related to the aging or to the origin were not specified, the aim of the study was the differentiation of wine vinegar categories and origin by the simplest and fastest method. So, the promising results obtained by the classification models, and even by the direct observation of the spectra, avoids the use of quantitative methods that need to know the specific compounds related to aging or origin, which would require trained operators as well as the use of standards and time-consuming analyses. In conclusion, the results demonstrated that the hierarchical classification model developed in this study with UV–vis spectra in conjunction with SIMCA and PLS-DA open up the possibility of developing a software that provides an easy and fast differentiation for the authentication of wine vinegars. Besides, this procedure present the possibility of in situ and non-destructive analysis using a portable instrument, which could be used as an alternative tool for PDO council regulations to standard control procedures.

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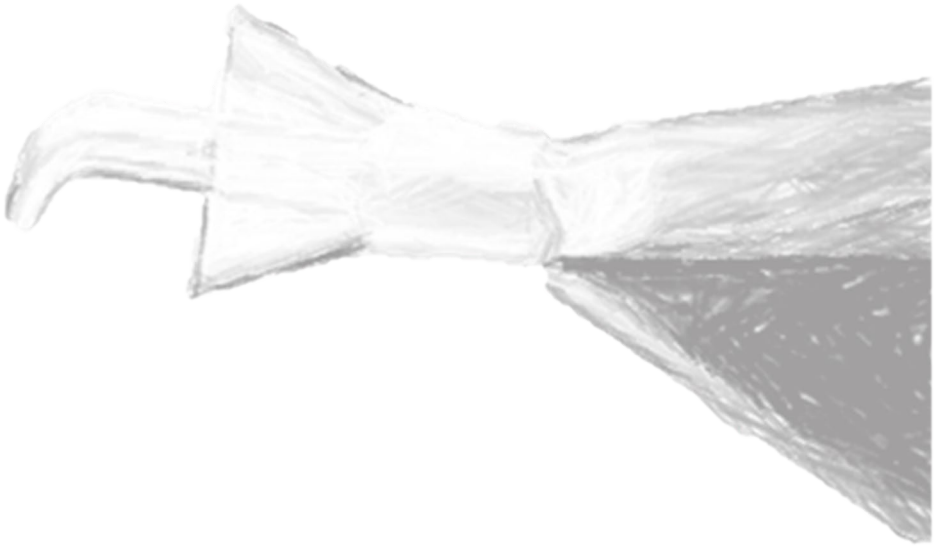

BLOQUE II:

CARACTERIZACIÓN ISOTÓPICA

CAPÍTULO V.

Estudio de la huella dactilar de isótopos C – O de vinagres de vino españoles con diferentes procedencias geográficas

CHAPTER V.

Study of C-O isotope fingerprint for Spanish wine vinegars from different geographical provenances

RESUMEN

El análisis de isótopos estables de Carbono y Oxígeno ($\delta^{13}C$ y $\delta^{18}O$) es uno de los métodos oficiales de control de los vinagres de vino. Por este motivo, este trabajo, publicado en European Food Research and Technology (2018) 244: 1159, tuvo como objetivo el análisis isotópico de los vinagres de vino con DOP por espectrometría de masas para relaciones isotópicas (IRMS) para el control de su autenticidad, así como para ver si los valores obtenidos pudieran utilizarse como huella digital de su origen geográfico en comparación con otras regiones.

Para ello se analizó, en primer lugar, un total de 35 muestras de vinagre de vino: 27 muestras de vinagres de vino proporcionadas por los Consejos Reguladores de las tres DOP de vinagres españoles “Vinagre de Condado de Huelva”, “Vinagre de Jerez” y “Vinagre de Montilla-Moriles", y 8 vinagres sin DOP del norte de España (Galicia, Cataluña y La Rioja) con procedencia garantizada. Todas estas muestras pertenecían al mismo año de producción (2014) y al mismo tiempo de envejecimiento (de 6 a 12 meses). En segundo lugar, para probar la posible variación de $\delta^{18}O$ entre años de cosecha y entre diferentes tiempos de envejecimiento en los vinagres con DOP, se analizó un segundo conjunto de vinagres de vino con DOP compuesto por 24 muestras, en los que se incluían vinagres del 2015, y vinagres con envejecimiento entre 6 y más de 12 meses de ambos años de producción.

La mayoría de los vinagres de vino españoles con DOP presentaron valores de $\delta^{13}C$ dentro de los valores de referencia marcados por estudios isotópicos previos sobre vinagres de vino europeos, ya que todas las muestras estaban por debajo del valor de referencia de -20 ‰. Este análisis es útil para indicar si el ácido acético y los azucares proceden verdaderamente de la uva (planta de tipo C3), o son obtenidos por la fermentación de otros productos como cereal, remolacha o caña de azúcar (plantas de tipo C4), los cuales mostrarían valores $\delta^{13}C$ entre -9 ‰ y -19 ‰. De ser así, estaríamos ante un caso de posible adulteración.

Por otra parte, la determinación de $\delta^{18}O$ confirmó su utilidad para discriminar a todos los vinagres españoles según las tres coordenadas geográficas (latitud, longitud y altitud). Los valores positivos de $\delta^{18}O$ se asociaron a vinagres del sur (2,16 ± 1,59 ‰) y los valores negativos se relacionaron con vinagres del norte (-2,93 ± 2,82 ‰). También se encontró una correlación significativa ($p < 0,001$) de $\delta^{18}O$ con altitud ($R2 = -0,48$) y longitud ($R2 = 0,36$), lo que permitió concluir que el análisis $\delta^{18}O$ podría ser utilizado como huella dactilar del origen geográfico en los vinagres de vino españoles. Así, los vinagres de la DOP Vinagre de Condado de Huelva mostraron los valores más altos de $\delta^{18}O$, seguidos de las muestras de DOP Vinagre de Jerez y Vinagre de Montilla-Moriles, así como los vinagres de Cataluña, Galicia y La Rioja mostraron

valores $\delta^{18}O$ negativos también significativamente diferentes entre sí. Esto puede deberse a que este valor se ve afectado por la fuente u origen del agua. Además, el análisis isotópico de $\delta^{18}O$ también ha demostrado ser capaz de detectar la adición de agua externa no proveniente de las uvas, usado para reducir el grado acético de forma fraudulenta. Si las muestras fuesen diluidas mostrarían valores de $\delta^{18}O$ de -5 ‰, mientras que en los vinagres de vino no adulterados sus valores rondarían el -2 ‰. Por último, aunque el tiempo de envejecimiento parecía aumentar los valores de $\delta^{18}O$, las diferencias de estos valores se mantuvieron entre las DOPs, concluyendo que las diferencias isotópicas marcan el origen geográfico por encima del tiempo de envejecimiento o el año de producción.

En conclusión, los resultados de este estudio confirman que los análisis de isótopos estables de oxígeno y carbono, especialmente el primero, podrían ser considerados como un método analítico útil para las autoridades reguladoras para identificar o evaluar el origen geográfico de los vinagres españoles y verificar el etiquetado correcto de la denominación geográfica, además de ser usados para controles oficiales rutinarios, que es para lo que hoy en día se utilizan. Sin embargo, todavía hay mucho trabajo por hacer, siendo interesante continuar con la caracterización isotópica de los vinagres de vino de alta calidad certificados incluyendo más muestras, con el fin de definir mejores rangos y límites y poder así desarrollar aplicaciones específicas.

ARTÍCULO 7

A viability study of C–O isotope fingerprint for different geographical provenances of Spanish wine vinegars

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ORIGINAL PAPER

A viability study of C–O isotope fingerprint for different geographical provenances of Spanish wine vinegars

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Abstract
Wine vinegar is an increasingly appreciated product in Europe and some high-quality vinegars have been certified with "Protected Designation of Origin" (PDO) to preserve and control their production methods. Spain has three of the five PDO wine vinegars existing in Europe. A tentative study was carried out to assess the utility of stable isotope analysis ($\delta^{13}C$ and $\delta^{18}O$) for the characterization of those Spanish wine vinegars and if the values obtained could be used as a fingerprint of their geographical origin compared with other regions. A total of 35 wine vinegar samples, belonging to the three Southern Spain PDOs and three Northern Spain non-PDO regions, were analyzed for their isotopic composition. Our analysis revealed that most of the Spanish vinegars presented $\delta^{13}C$ values that were in agreement with some other isotopic studies about Mediterranean vinegars, since all the samples were under $-20‰$ and the vast majority of them were below $-24‰$. On the other hand, the $\delta^{18}O$ analysis confirmed its utility for discriminating all the Spanish vinegars according to the three geographical coordinates (latitude, longitude and altitude). Positive mean values were associated to Southern vinegars ($2.16 \pm 1.59‰$) and negative $\delta^{18}O$ values were related with Northern vinegars ($-2.93 \pm 2.82‰$). We also found a highly significant ($p < 0.001$) correlation of $\delta^{18}O$ with altitude ($R^2 = -0.48$) and longitude ($R^2 = 0.36$), which allowed us to conclude that $\delta^{18}O$ analysis could be used as a fingerprint of the geographical origin in Spanish wine vinegars.

Keywords Wine vinegar · Protected designation of origin · Stable isotopes ratio · ^{13}C · ^{18}O · Geographical origin

Introduction

Vinegar is a food product consumed worldwide as a condiment and food-preserving agent, which can be produced by different methods and raw materials (such as malt, apple, rice, etc.). The legal definitions of vinegar vary from one country to another. The term "vinegar" can be described a product of a double fermentation (alcoholic followed by acetous fermentation) from substances of agricultural origin [1]. Within the wide range of vinegar types, wine vinegar is the most commonly produced and consumed vinegar in Mediterranean countries and Central Europe [2]. This product is the result of the conversion of must sugars into ethanol by the action of yeasts, and the subsequent ethanol oxidation by acetic acid bacteria [3]. In fact, in accordance with [4] (Annex VII, Part II), an authentic wine vinegar cannot contain synthetic acetic acid or acetic acid from the fermentation of sugars that are not derived from grapes (e.g., derived from beet or cane). Furthermore, authentic wine vinegars cannot be produced from dried grapes diluted with water [5, 6]. In that sense, the main producers of wine vinegars as Italy or Spain consider vinegar as a product obtained from acetous fermentation of wine [1].

For many years, wine vinegar has been considered as a low-cost secondary product spontaneously derived from wine production. However, in recent years, wine vinegar has become a highly-appreciated food commodity in gastronomy [7]. As a result, the demand for high-quality wine vinegars has significantly increased over the last years. In this context, Spain is one of the major producers of high-quality wine vinegars, including three of the five types of

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vinegar registered in Europe [8] with a "Protected Designation of Origin" (PDO): "Vinagre de Jerez" (also known as "Sherry wine vinegar"), "Vinagre de Montilla-Moriles" and "Vinagre de Condado de Huelva". The production of these high-quality PDO wine vinegars in Spain is centered in Andalusia, a Southern Spanish region traditionally associated to wine culture, and each of them is produced using the corresponding protected wines ("Vino de Jerez", "Vino de Montilla-Moriles" and "Vino de Condado de Huelva"), which provide singular and specific characteristics. These vinegars have high prices in the market due to their high quality, the long aging time in wooden butts and the high cost of their production. Their high price increases the vulnerability of these products to fraud [9]. For this reason, impartial tools are required to fight against mislabeling or even falsification and better systems must be established to define their quality, authenticity and geographical origin. In many cases, an irrefutable conclusion about the authenticity of a sample using conventional methods is not possible and more accurate information is needed for obtaining a high guarantee of authentication. With this purpose, the analysis of isotope ratios of some bioelements is being evaluated to provide a geographical profiling of food products that can be applied in geographical identification [1]. In this regard, the analysis of stable isotope ratios of carbon ($^{13}C/^{12}C$; expressed as $\delta^{13}C$) and oxygen ($^{18}O/^{16}O$; expressed as $\delta^{18}O$), among others, has already been introduced as an officially accepted method in food authenticity and origin determination [10]. Since 1991, the addition of water and exogenous sugars (from beet or sugarcane), the most common adulterations of wine, has been detected in wine by analyzing the isotopic ratios of hydrogen (D/H) and carbon ($^{13}C/^{12}C$) in ethanol and of oxygen ($^{18}O/^{16}O$) in water. International Organization of Vine and Wine (OIV) methods are currently adopted: OIV-MA-AS311-05 for site-specific analysis of the D/H ratio using ^{2}H-site-specific natural isotope fractionation NMR (^{2}H-SNIF-NMR), OIV-MA-AS312-06 for analysis of the $^{13}C/^{12}C$ ratio (expressed as $\delta^{13}C$ ‰) using isotope ratio mass spectrometry (IRMS), OIV-MA-AS2-12 for analysis of the $^{18}O/^{16}O$ ratio (expressed as $\delta^{18}O$‰) using IRMS. Very recently, isotopic methods have been recognized by the European Committee for Standardization (CEN) and in part by OIV as a means of detecting the presence of exogenous acetic acid and tap water in wine vinegar [5, 6].

The $\delta^{13}C$ of the acetic acid can indicate if the source of the acetic acid and the grape sugars is truly grape (wine) ethanol or wine must, or other ethanol made from fermentation of some other agricultural products such as cereal, potato starch, beetroot or sugarcane. Concerning photosynthetic pathway, some of those plants are C_4-type (Hatch–Slack, C4-dicarboxylic acid pathway), whose $\delta^{13}C$ values commonly range from -9 to -19‰. By contrast, these values usually range from -20 to -35‰ in vine and the rest of C_3-type plants, in which the atmospheric CO_2 is fixed through the reductive pentose phosphate pathway [11]. Since ^{13}C analysis by isotope ratio mass spectrometry (IRMS) has shown a strong capability to identify synthetic vinegars and distinguish and detect photosynthetic C_3-type (as grape) and C_4-type (as sugarcane or maize)-derived products in mixtures, it could be used as a tool for the detection of adulterated vinegars [1]. Although the photosynthetic pathway defines the carbon isotope ratios of plant in its organic matter [12, 13], some environmental factors or conditions, as water stress, can also cause these values to increase or decrease during the growing cycle, resulting in detectable differences even within the same genotype [14]. Furthermore, it must be noted that the isotopic values of acetic acid are not affected when the methods for transforming wine to vinegar are applied [6]. Regarding $\delta^{18}O$, the value for this isotope is primarily affected by the source of plant-available water. Chiocchini et al. [15] reported that lower values of $\delta^{18}O$ in extra-virgin olive oils were related to water from regions with high elevation, inland location and cool climate, whereas higher values were related to areas with low elevation, coastal location and warmer weather. Similar studies carried out in wine also found that its isotopic composition is significantly determined by the climate conditions during the pre-vintage period [16, 17]. In general terms, the ^{18}O content of the water in grape products has shown to depend on the environment—natural or anthropogenic—from which it originates. Thus, the isotopic ^{18}O analysis has also shown to be able to detect the addition of external water, not coming from the grapes. The possible dilutions with tap water to reduce the acetic degree in the resulting wine vinegar could be detected since this practice leads to significant changes in the ^{18}O isotopic ratio [1]. Thereby, Thomas and Jamin [18] demonstrated the potential of the oxygen-stable isotope analysis of water to distinguish wine vinegar from vinegars made from dried grapes, where the ^{18}O isotopic content is influenced by the tap water used in production. Furthermore, Camin et al. [5] proved experimentally that $\delta^{18}O$ analysis of beverage water, officially used to detect the watering of wine and rehydration of concentrated fruit juice, can also be applied to vinegar to detect this kind of fraud. They established minimum values for the $\delta^{18}O$ of water in -2 and -5‰ for raw and diluted vinegars, respectively. $\delta^{18}O$ values lower than -5‰ in wine vinegar products, therefore, could indicate an anomalous and excessive water addition.

The fact that water source affects $\delta^{18}O$ values makes the latter eligible to be selected as potential geographical markers of vinegars. Thus, the legal limits established for wine, which are based on the wine isotope databank, have been used as reference for $\delta^{18}O$ analysis to detect the geographical origin authenticity of wine vinegar [6]. Raco et al. [11] also confirmed the efficacy of $\delta^{18}O$ analysis, together with the determination of the deuterium (^{2}H) isotope content, for

detecting the geographical origin of wine. However, since the ^{2}H and ^{18}O isotopic compositions of those products showed a high correlation in that research, $\delta^{2}H$ value could be considered a dependent value from the $\delta^{18}O$ value.

Despite the promising results, much work is needed since these types of studies are still scarce and had not previously been made with Spanish PDO. Therefore, it would be interesting to continue with the isotopic characterization of certified high-quality wine vinegars, to develop specific applications. The aim of this work was to characterize Spanish wine vinegars from different regions with respect to their oxygen and carbon isotopic ratio and to assess the applicability of these values to determine their geographical origin. This tentative study also aimed to provide a better understanding of the isotopic composition of wine vinegars and its correlation with climatic conditions, water content of wine, production process and origin.

Materials and methods

Wine vinegar samples

27 samples provided by the Regulatory Councils of the three Andalusian PDOs "Vinagre de Condado de Huelva" (11), "Vinagre de Jerez" (11) and "Vinagre de Montilla-Moriles" (5) were selected and analyzed. Furthermore, eight samples belonging to commercial vinegars from Northern Spain—Galicia (2), Catalonia (3) and La Rioja (3)—with a guaranteed provenance were included. Further information about grape variety, year of harvest, acetic acid degree and geographical location can be found in Table 1.

The number of samples per group is not well balanced, being the Northern group considerably smaller than the Southern group of samples, although it was in accordance to the market availability of vinegars in Spain. Thus, the rate of production and, therefore, commercialization of wine vinegars in Spain is higher for those with PDO due to their high quality provided by their certification. Hence, the total number of wine vinegar samples collected from Southern Spain, registered under a PDO, has been higher than those samples from Northern regions due to the high demand, and therefore high presence of the former. Moreover, to ensure the provenance of the samples without a PDO (Northern wine vinegars), only wine vinegars produced from wines of high-quality made with the typical varieties of geographical area were selected. This led to reduce the number of northern samples available but guaranteed its origin and quality. The year of harvest of the grape used to setup the vinegars of this set of samples was the same (2014) to compare samples of the same vintage and to avoid changes in the ^{18}O ratio due to this factor. Furthermore, the time of aging in wooden butts was also considered in the criteria selection of these samples, due to it is a factor that could have an isotopic fractionation effect. For this reason, all samples of this first set were aged for a short and similar aging period (between 6 and 12 months).

In addition, to test the possible variation of ^{18}O ratio between years of harvest and among different aging times, a second set of Spanish PDO vinegars were analyzed: 9 "Vinagre de Condado de Huelva" samples (6 from 2015: 3 with more than 12 months of aging and 3 with less than 12 months; and 3 from 2014 with more than 12 months), 11 "Vinagre de Jerez" samples (7 from 2015: 4 with more than 12 months of aging and 3 with less than 12 months; and 4 from 2014 with more than 12 months) and 4 "Vinagre Montilla-Moriles" samples (2 from 2015: 1 with more than 12 months of aging and other with less than 12 months; and 2 from 2014 with more than 12 months) (Table 2).Samples were analyzed in triplicates.

Isotopic analysis

The $^{13}C/^{12}C$ and $^{18}O/^{16}O$ results are expressed in standard delta notation (δ) as per mil (‰) deviation from the standards VPDB (Vienna-Pee Dee Belemnite, IAEA, Vienna) normalized by assigning consensus values [19], and V-SMOW (Vienna-Standard Mean Ocean Water) normalized to the VSMOW–SLAP (Standard Light Antarctic Precipitation) scale, respectively, according to the equation:

$$\delta\,(i/j\,E)\,‰ = \frac{i/j\mathrm{Rp} - i/j\mathrm{RRef}}{i/j\mathrm{RRef}} \times 1000$$

where ${}^{i/j}E$ denotes the higher (superscript i) the lower (superscript j) atomic mass number of element E and R is the ratio of the heavy to light stable isotope in the sample (R_p) and the international reference material (R_{ref}) = $^{18}O/^{16}O$ or $^{13}C/^{12}C$ ratios.

$\delta^{13}C$ determination was performed by combusting the sample at 1020 °C in a Carlo Erba 1108 elemental analyzer coupled in continuous flow mode to an IRMS (Isotope Ratio Mass Spectrometer) VG Isochrom. The main standards used were NBS-22, IAEA CH6 and IAEA 600. The analytical precision, based on the repeated analysis of internal standard waters, was 0.1‰.

$\delta^{18}O$ determination was conducted by a process of equilibration with a mixture of gases He–CO_2 for 18 h and further analysis of CO_2 in a GasBench coupled in continuous flow mode to an Isotope Ratio Mass Spectrometer (IRMS) Delta V Advantage, Bremen (Germany). The main standards used for correction were V-SMOW2 and SLAP2. Measurement precision (on the standards used) was 0.2‰.

All measurements were carried out against laboratory standards that are periodically calibrated against

Table 1 Characteristics of the samples according to grape variety, geographical location and isotopic ^{18}O and ^{13}C ratios

PDO/origin	No	Grape variety	Acetic degree	Spatial characteristics			$\delta^{13}C‰$ vs. VPDB	$\delta^{18}O‰$ vs. VSMOW
				Latitude	Longitude	Altitude (m)		
Condado de Huelva	1	Zalema	8.0	37°22′01″N	06°32′29″W	192	− 26.2	1.8
	2		8.0	37°22′01″N	06°32′29″W	192	− 25.2	1.5
	3		8.0	37°22′01″N	06°32′29″W	192	− 25.0	2.4
	4		7.0	37°22′01″N	06°32′29″W	192	− 25.2	2.4
	5		8.0	37°22′01″N	06°32′29″W	192	− 25.2	6.8
	6		8.0	37°22′01″N	06°32′29″W	192	− 22.3	2.3
	7		8.0	37°22′01″N	06°32′29″W	192	− 25.1	2.0
	8		8.0	37°22′01″N	06°32′29″W	192	− 24.5	1.9
	9		8.0	37°22′01″N	06°32′29″W	192	− 25.1	2.3
	10		8.0	37°22′01″N	06°32′29″W	192	− 24.4	2.8
	11		7.0	37°22′01″N	06°32′29″W	192	− 24.9	3.1
Jerez	1	Palomino	7.0	36°36′58″N	06°09′08″W	20	− 24.4	0.3
	2		7.0	36°42′00″N	06°07′00″W	56	− 24.4	0.6
	3		7.0	36°42′00″N	06°07′00″W	56	− 25.6	2.4
	4		7.0	36°43′08″N	06°19′48″W	32	− 24.6	1.3
	5		7.0	36°36′58″N	06°09′08″W	20	− 24.8	2.3
	6		7.0	36°43′08″N	06°19′48″W	32	− 25.3	3.2
	7		7.0	36°36′58″N	06°09′08″W	20	− 25.1	0.7
	8		7.0	36°42′00″N	06°07′00″W	56	− 25.6	3.5
	9		7.0	36°42′00″N	06°07′00″W	56	− 25.0	2.8
	10		7.0	36°42′00″N	06°07′00″W	56	− 26.1	1.3
	11		7.0	36°36′58″N	06°09′08″W	20	− 25.0	2.5
Montilla-Moriles	1	Pedro Ximénez	8.0	37°36′10″N	04°38′03″W	207	− 24.2	− 0.5
	2		8.0	37°36′10″N	04°38′03″W	207	− 26.1	2.4
	3		9.7	37°36′10″N	04°38′03″W	207	− 25.4	5.8
	4		7.0	37°36′ 10″ N	04°38′03″W	207	− 24.9	− 0.1
	5		6.0	37°29′ 53″N	04°25′51″W	547	− 23.3	0.3
Galicia	1	Albariño	6.0	42°07×59″N	08°15×47″W	187	− 25.3	− 2.2
	2		6.0	42°07×59″N	08°15×47″W	187	− 22.2	− 3.6
Catalonia	1	Chardonnay Blanc	6.0	41°22′50″N	01°36′39″E	257	− 25.6	− 1.4
	2		6.5	41°22′50″N	01°36′39″E	257	− 24.7	1.9
	3		6.0	41°22′50″N	01°36′39″E	257	− 25.5	− 0.8
La Rioja	1	Viura	6.0	42°28′12″N	02°26′44″W	465	− 23.9	− 5.9
	2		6.0	42°28′12″N	02°26′44″W	465	− 25.3	− 5.2
	3		6.0	42°28′12″N	02°26′44″W	465	− 23.7	− 6.2

All samples had the same year of harvest (2014) and were aged less than 12 months

international standards recommended by the International Atomic Energy Agency (IAEA).

Statistical analysis

The isotopic data were analyzed using Infostat software (Grupo InfoStat, Argentina). Analysis of variance (ANOVA) and least significant difference (LSD) Fisher test were carried out on the data set to identify differences between groups of samples from certain geographical Spanish origin. The Pearson correlation coefficient (R) and p value were used to show the linear dependence and their significance between the studied variables. Probability values of $p < 0.05$ were adopted as the criterion for significant differences.

Table 2 Isotopic ^{18}O and ^{13}C ratios of a new set of PDO wine vinegars including different aging time and different years of harvest

PDO/origin	No	Grape variety	Acetic acid	Year	Spatial characteristics			Aging (months)	$\delta^{13}C‰$ vs. VPDB	$\delta^{18}O‰$ vs. VSMOW
					Latitude	Longitude	Altitude			
Condado de Huelva	12	Zalema	10	2015	37°22′01″N	06°32′29″W	192	< 12	− 26.4	2.5
	13		7	2015	37°22′01″N	06°32′29″W	192	< 12	− 26.2	2.2
	14		8	2015	37°22′01″N	06°32′29″W	192	< 12	− 25.5	3.7
	15		8	2014	37°22′01″N	06°32′29″W	192	> 12	− 25.4	5.9
	16		8	2014	37°22′01″N	06°32′29″W	192	> 12	− 25.2	1.7
	17		8	2015	37°22′01″N	06°32′29″W	192	> 12	− 24.5	2.3
	18		6	2015	37°22′01″N	06°32′29″W	192	> 12	− 25.4	4.7
	19		8	2015	37°22′01″N	06°32′29″W	192	> 12	− 25.2	1.5
	20		6	2014	37°22′01″N	06°32′29″W	192	> 12	− 24.4	5.9
Jerez	12	Palomino	7	2015	36°36′58″N	06°09′08″W	20	< 12	− 24.9	0.6
	13		7	2015	36°42′00″N	06°07′00″W	56	< 12	− 24.9	2.1
	14		7	2015	36°36′58″N	06°09′08″W	20	< 12	− 25.1	3.0
	15		7	2015	36°40′43″N	06°09′13″W	20	< 12	− 24.9	0.7
	16		8	2014	36°43′08″N	06°19′48″W	32	> 12	− 25.1	3.7
	17		7	2015	36°36′58″N	06°09′08″W	20	> 12	− 24.9	3.7
	18		8.3	2014	36°42′00″N	06°07′00″W	56	> 12	− 25.8	3.9
	19		7	2014	36°42′00″N	06°07′00″W	56	> 12	− 25.7	2.1
	20		7	2015	36°42′00″N	06°07′00″W	56	> 12	− 24.3	2.2
	21		7	2014	36°40′43″N	06°09′13″W	20	> 12	− 25.2	2.0
	22		9.5	2015	36°40′43″N	06°09′13″W	20	> 12	− 24.9	4.6
Montilla-Moriles	6	Pedro Ximé-nez	8	2015	37°36′10″N	04°38′03″W	207	< 12	− 24.7	1.4
	7		8	2014	37°36′10″N	04°38′03″W	207	> 12	− 25.0	3.7
	8		8	2014	37°36′10″N	04°38′03″W	207	> 12	− 24.4	1.4
	9		8	2015	37°36′10″N	04°38′03″W	207	> 12	− 25.7	3.4

Results and discussion

$\delta^{13}C$ and $\delta^{18}O$ isotopic values in different Spanish wine vinegars

Isotopic ^{18}O and ^{13}C ratios vs. each corresponding standard obtained in the analysis of the different wine vinegar samples are shown in Tables 1 and 2. Regarding those results, $\delta^{13}C$ values ranged from −26.4 (corresponding to a sample from "Vinagre de Condado de Huelva" PDO) to −22.2‰ (corresponding to a sample from "Galicia"), whereas $\delta^{18}O$ ranged from −6.2 (corresponding to a sample from La Rioja) to 6.8‰ (corresponding to a sample from "Vinagre de Condado de Huelva" PDO). With respect to ^{13}C isotope, several researches have demonstrated the association between $\delta^{13}C$ values, and wine and wine vinegar adulterations due to the relationship of these values with the photosynthetic pathway. According to that, Raco et al. [11] claimed that the $\delta^{13}C$ (vs. VPDB) values from wine ethanol, that has been found to be not different from that of acetic acid after extraction from vinegars, should range from −20 to −35‰. By contrast, some other authors [1, 6, 20] established that values between −24 and −20‰ in temperate European regions have shown to be related to vinegars with acetic acid coming from C_4 plants or from grapes exposed to severe water stress. In regards to the results obtained in this study (Tables 1, 2), we found that all our samples presented values less than −20‰ and that the vast majority of them were below the threshold of −24‰.

With respect to the determination of stable oxygen isotope ratio, Camin et al. [5] established the minimum value of the $\delta^{18}O$ for raw vinegars (−2‰) and diluted vinegars (−5‰), which was also confirmed by Werner and Roβmann [1]. According to that, our results showed that most of the vinegar samples were in agreement with those authors' findings, with only two samples below −2‰ and three below −5‰ (Tables 1, 2). Besides, almost all of the samples belonging to the three Andalusian PDOs presented positive values of $\delta^{18}O$.

As a visual summary of the aforementioned results, Fig. 1 depicts the isotopic $\delta^{13}C$ and $\delta^{18}O$ values of all the samples considered in the present study, together with the aforementioned thresholds, separating samples according to their isotopic ^{13}C and ^{18}O content. As shown in the Fig. 1, the vinegars from the three Andalusian PDOs are placed together in the same area of the graph, regardless of the year of harvest and the aging time. In general terms, it can be observed that vinegar samples from "Vinagre de Jerez" PDO had the most homogeneous isotopic values (SD values of ±0.54 and ±1.11 for $\delta^{13}C$ and $\delta^{18}O$, respectively), followed by "Vinagre de Condado de Huelva" (±0.95 and ±1.45), possibly due to the fact that these PDOs are highly consolidated. Samples from "Vinagre de Montilla-Moriles" PDO, however, presented a higher dispersion (±1.07 and ±2.62) within their isotopic values (Table 3). This recent PDO was registered in 2015 and that fact, together with the difficulty

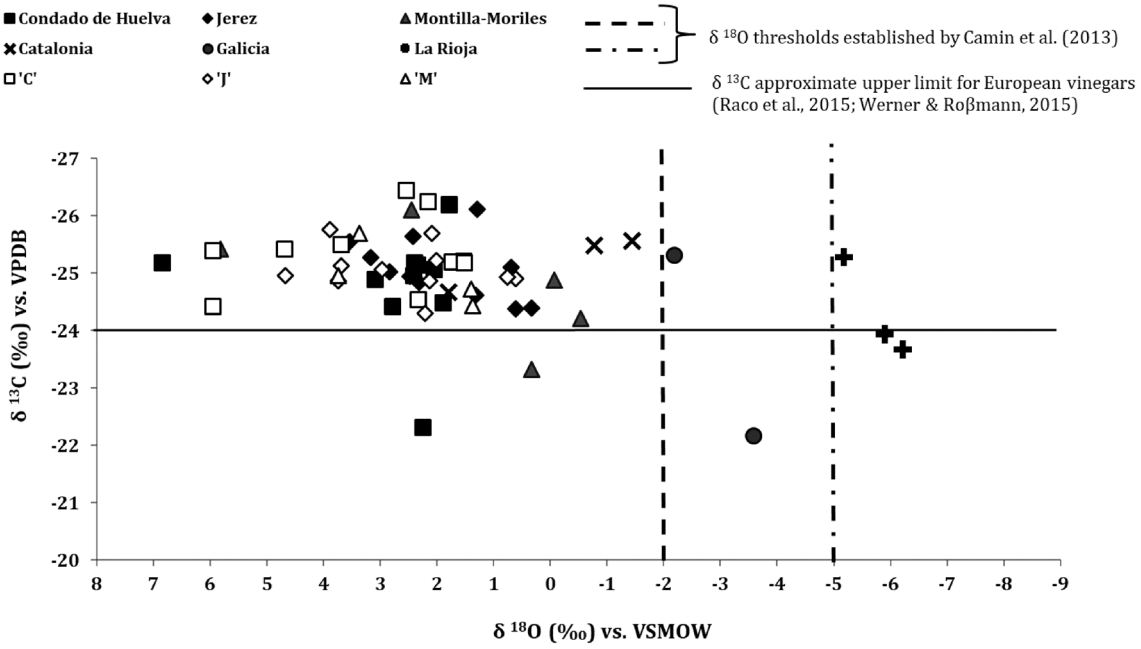

Fig. 1 $\delta^{13}C$ values and $\delta^{18}O$ values from different Spanish wine vinegars. Condado de Huelva 'C', Vinagre de Jerez 'J' and Montilla Moriles 'M' vinegars from 2015 and more than 12 months of aging, respectively (Table 2)

Table 3 Mean values ± standard deviation (SD) and ANOVA results of isotopic ^{13}C and ^{18}O ratios (‰) according to geographical origin

Isotopic ratios	Geographical provenance	Mean value ± SD (‰)
$\delta^{13}C$ (‰) vs. VPDB	Galicia	-23.74 ± 2.23^{a}
	La Rioja	$-24.30 \pm 0.86^{a,b}$
	Montilla-Moriles	$-24.79 \pm 1.07^{b,c}$
	Condado de Huelva	$-24.82 \pm 0.95^{b,c}$
	Jerez	-25.08 ± 0.54^{c}
	Catalonia	-25.24 ± 0.50^{c}
$\delta^{18}O$ (‰) vs. VSMOW	Condado de Huelva	2.67 ± 1.45^{a}
	Jerez	$1.91 \pm 1.11^{a,b}$
	Montilla-Moriles	1.60 ± 2.62^{b}
	Catalonia	-0.12 ± 1.75^{c}
	Galicia	-2.89 ± 0.99^{d}
	La Rioja	-5.77 ± 0.53^{e}

Letters mean significant differences ($p < 0.05$) according to LSD test

in obtaining a higher number of samples in the present study, could explain the obtained results [21, 22]. Wine vinegars with a PDO indication are exposed to an exhaustive routine checked by the Regulatory Councils that largely prevent the occurrence of possible production frauds and errors. This is a quality guarantee confirmed by our results.

Isotopic differentiation of geographical origin

To determine whether the $\delta^{13}C$ and $\delta^{18}O$ change according to the geographical origin, two sets of $ANOVA_s$ and LSD Fisher tests were performed for each isotope. One set consisted on six different regions of Spain where wine vinegars are produced (Condado de Huelva, Jerez, Montilla-Moriles, Galicia, La Rioja and Catalonia) and the second set included those Spanish regions grouped in two classes according to latitude: Northern and Southern Spain. ANOVA results are shown in Table 3. With regard to $\delta^{13}C$, significant differences ($p < 0.05$) according to geographical origin and latitudes North and South were observed. The significant differences between North and South (-24.51 ± 1.19‰ for Northern vinegars and -24.92 ± 0.81‰ for Southern vinegars) were not very evident. Moreover, this parameter is more related to adulteration detection and no clear differentiation of the PDO zone is achieved. The level of discrimination was higher within $\delta^{18}O$ values. In fact, mean values of $\delta^{18}O$ obtained for the North (-2.93 ± 2.82‰) and South locations (2.16 ± 1.59‰) showed greater significant differences than $\delta^{13}C$ mean values. Furthermore, with respect to $\delta^{18}O$ results, positive mean values were associated to Southern vinegars ("Vinagre de Condado de Huelva", "Vinagre de Jerez" and "Vinagre de Montilla-Moriles" in this order) and negative $\delta^{18}O$ values were related with Northern vinegars (Table 3). The significant variability observed within $\delta^{18}O$ values reflected the effect of geographical origin and its associated meteorological conditions on the isotopic composition of wine vinegars, separating the considered samples according to Southern and Northern regions. Hence, the isotopic results obtained in this study were in agreement with other researches that demonstrated the utility of isotopic analysis in the determination of geographical origin of butter [23], the discrimination of milks produced at different altitudes [24] and the influence of climatic conditions and grape variety, highly related to geographical origin, in vinegars and wines [6, 11].

With respect to samples from PDOs, the $\delta^{18}O$ mean value for "Vinagre de Condado de Huelva" samples were the highest (2.67 ± 1.45‰), while these values were significantly lower for "Vinagre de Montilla-Moriles" wine vinegars (1.60 ± 2.62‰). The values of "Vinagre de Jerez" samples were between these two extremes (1.91 ± 1.11‰). These intermediate values may be associated to the geographical origin of these samples, since the production area of "Vinagre de Jerez" PDO wine vinegars is located between "Vinagre de Condado de Huelva" and "Vinagre de Montilla-Moriles" PDOs (all of them in Southern Spain), being the land type and the latitude and longitude coordinates very similar (Table 1). These characteristics make their differentiation with only $\delta^{18}O$ isotopic analysis difficult to accomplish.

On the other hand, ANOVA was also performed to determine whether the $\delta^{13}C$ and $\delta^{18}O$ change according to time of aging in wood barrels and the year of harvest. Regarding the aging time factor, any significant differences were not observed for $\delta^{13}C$ isotopes. However, with regards to $\delta^{18}O$ isotope, it was possible to observe for each PDO that samples aged more than 12 months had higher values, incrementing around 1.5 times with respect to less aged samples (Table 2). In spite of this, the $\delta^{18}O$ mean value for "Vinagre de Condado de Huelva" samples aged for more than 12 months was the highest (3.69 ± 2.08‰) as it was observed before, "Vinagre de Jerez" aged samples had also intermediate values (3.18 ± 1.06‰) and the $\delta^{18}O$ mean values for "Vinagre de Montilla-Moriles" more aged vinegars were again significantly lower (2.82 ± 1.27‰). Furthermore, these values were even more significantly different from those of Northern vinegars due to $\delta^{18}O$ mean values for this group was negative (-2.93 ± 2.82‰). Regarding the year of harvest, and taking into account only samples for the same category (< 12 months) to avoid the variance showed with the aging factor, $\delta^{13}C$ and $\delta^{18}O$ mean values did not show significant differences between the 2 years of harvest considered for the three PDOs, mainly due to the complex production process of these vinegars, that had many processes between the harvest of the grapes and the final product, including, among them, their aging in wood barrels. Thus,

$\delta^{13}C$ mean values in 2014 and 2015 for this isotope were around -25.0 ± 0.16‰ and -25.20 ± 0.70‰, respectively, as well as mean values in 2014 and 2015 for $\delta^{18}O$ ratio were around 2.00 ± 0.5, 1.94 ± 0.70‰, respectively. Hence, these results showed that even with different years of harvest or aging periods, the ^{13}C and ^{18}O ratios were mainly dependent of the geographical origin.

Finally, a study of correlations between the isotopic ratios and some vinegar parameters described in Table 1 was carried out. The Pearson's correlation coefficients, shown in Table 4, showed that there is a highly significant ($p < 0.001$) correlation of the isotopic ^{18}O ratio with geographical location expressed as altitude (samples from regions with different elevation above sea level), longitude (samples from Eastern and Western regions) and latitude (samples from Southern and Northern regions). From these three geographical variables, the higher correlation was observed in latitude (− 0.77). This result was in agreement with the conclusions of Renou et al. [24] on their research about milk and with those from Chiocchini et al. [15] for extra-virgin olive oils, who claimed that lower values of $\delta^{18}O$ were related to waters from regions with high elevation, inland location and cool climate. Consequently, and according to the obtained results, the $\delta^{18}O$ determination showed to be more useful for the discrimination of wine vinegar according to its origin than the $\delta^{13}C$, since ^{18}O ratio was correlated with the three geographical coordinates or parameters, whereas $\delta^{13}C$ was only correlated with the latitude ($p < 0.01$) and with a lower correlation coefficient (0.27 for $\delta^{13}C$ vs. − 0.77 for $\delta^{18}O$). This Pearson's correlation analysis was in agreement with the results obtained in the previous ANOVAs (Table 2) and reinforced the conclusions of the present study.

Conclusions

The results of the present study indicated that $\delta^{18}O$ analysis can be used as a valid tool to distinguish among some Spanish wine vinegars from different latitude, North and South (negative and positive $\delta^{18}O$ values respectively), and even among regions with similar latitude. "Vinagre de Condado de Huelva" PDO wine vinegars showed the highest $\delta^{18}O$ values, followed by "Vinagre de Jerez" and "Vinagre de Montilla-Moriles" PDO samples. In fact, the results showed that mean values of $\delta^{18}O$ between 2.67 and 1.60‰ could be established for Southern Spanish PDO wine vinegars. This range could be used as a fingerprint of these high-quality wine vinegars. Furthermore, the $\delta^{18}O$ isotopic values showed a significant correlation with different altitude, latitude and longitude.

The carbon stable isotope analysis revealed that most of the Spanish vinegars presented $\delta^{13}C$ values that were in agreement with some other isotopic studies about European wine vinegars.

According to the results, although the time of aging in wood barrels seems to increase the $\delta^{18}O$ isotope of PDO vinegars, the same the isotopic relationship and differences was maintained among the different vinegars. Therefore, we could conclude that the ^{13}C and ^{18}O ratios were mainly dependent of the geographical origin, even with different years of harvest or aging periods.

In conclusion, the results of this study allowed confirming that stable isotope ^{18}O and ^{13}C analysis, especially the first one, could be considered as a useful analytical method for regulatory authorities to identify or assess the geographical origin of Spanish wine vinegars and verify the correct labeling of geographical denomination. Further studies with higher number of samples, and some controlled samples would contribute to define better ranges and limits of isotope ratios for each area.

Table 4 Pearson's correlation coefficients among the different variables studied

Variables	Latitude	Longitude	Altitude	$\delta^{13}C$
Latitude				
Longitude	− 0.52***			
Altitude	0.54***	− 0.55***		
$\delta^{13}C$	0.27**	0.10^{NS}	0.17^{NS}	
$\delta^{18}O$	− 0.77***	0.36***	− 0.48***	− 0.35***

NS non-significant

*, **, ***Significant at 5, 1 and 0.1% levels, respectively

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Compliance with ethical standards

Conflict of interest The authors report no conflicts of interest.

Compliance with ethics requirements No humans nor animals were involved in the study.

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BLOQUE III.

CARACTERIZACIÓN Y CLASIFICACIÓN SENSORIAL DE LOS VINAGRES DE VINO ESPAÑOLES CON DOP

CAPÍTULO VI:

Caracterización del perfil volátil de los vinagres de vino españoles con DOP

CHAPTER VI.

Characterization of the volatile profile of Spanish PDO wine vinegars

RESUMEN

La calidad y composición aromática de los vinagres de vino con DOP viene determinada por la materia prima, el método de producción y los procesos de envejecimiento. Para estudiar el perfil volátil de una matriz alimentaria, como es el vinagre de vino, se utiliza la cromatografía de gases-espectrometría de masas (GC-MS), siendo necesaria una etapa previa de extracción. La técnica de extracción utilizada para su análisis puede influir positiva o negativamente en los resultados obtenidos. Por ello, en el primer trabajo de este capítulo, publicado en Food Research International 105 (2018) 880–896, se estudiaron y compararon las tres técnicas de muestreo o extracción que más se utilizan para matrices alimentarias, extracción por sorción en espacio de cabeza estático (HSSE), microextraccion en fase sólida en espacio de cabeza estático (HS-SPME) y extracción en espacio en cabeza dinámico (DHS), con el fin de seleccionar la mejor metodología de muestreo para la caracterización del perfil volátil de los vinagres de vino españoles con DOP. Además, este trabajo implementa una técnica quimiométrica útil para resolver problemas cromatográficos, denominada resolución de curvas multivariada (MCR), que permite extraer información relevante e identificar nuevos analitos, así como acelerar el procesamiento de datos.

Las condiciones de extracción de las tres técnicas de muestreo estudiadas se basaron en métodos previamente validados y publicados: HSSE según Callejón et al, 2008; DHS según Úbeda et al, 2016; y HS-SPME según Pizarro et al., 2008, Natera Marín et al., 2002 y Cirlini et al., 2001. Se seleccionaron 10 muestras de vinagre de vino pertenecientes a las tres DOP españolas y a dos categorías: Reserva, por ser una categoría intermedia entre los dos tiempos de envejecimiento, y Pedro Ximénez, por ser una de las categorías más presentes en el mercado.

El pre-procesado de los cromatogramas por MCR, además de reducir los problemas asociados al análisis GC-MS de mezclas complejas y facilitar el procesamiento de los datos, permitió determinar 81 compuestos volátiles, algunos de los cuales no se habían identificado anteriormente en vinagres de vino. Tras determinar dichos compuestos, se realizaron varios análisis de varianza (ANOVA) para determinar las diferencias significativas entre los métodos de muestreo empleados, las DOPs o las categorías consideradas. Los resultados mostraron que el método de muestreo era el que presentaba diferencias significativas entre un mayor número de compuestos. Seguidamente, se realizó un test Tukey que volvió a mostrar que la mayoría de los compuestos volátiles presentaban diferencias significativas entre técnicas de extracción, influyendo, por tanto, en gran medida en el perfil volátil resultante de cada muestra.

Además, para evaluar cómo influye el método de muestreo en la caracterización y diferenciación de diferentes tipos de vinagres de vino pertenecientes a diferentes DOP y categorías, se realizó un análisis de componentes principales (PCA).

Los resultados mostraron nuevamente que no todos los métodos de muestreo eran igual de adecuados para la caracterización y diferenciación entre DOP y categorías. Así, HSSE fue la técnica seleccionada debido a que extrajo un mayor número de compuestos para la mayoría de las familias químicas, y por tanto aportaba mayor información, así como en términos de diferenciación, fue la técnica, junto con HS-SPME, que mostró una mejor discriminación de las DOPs y categorías.

Por ello, una vez seleccionado HSSE como técnica de extracción, en el segundo trabajo que se presenta en este capítulo, publicado en Food Research International 123 (2019) 298–310, se analizó por HSSE-GC-MS en combinación con técnicas quimiométricas, un mayor número de vinagres de vino con DOP considerando todas sus categorías comercializadas. Por tanto, en este segundo trabajo, por primera vez se realiza un estudio detallado y comparativo del perfil volátil de estos vinagres de vino, usando el mismo método analítico (HSSSE-GC-MS) con el objetivo de intentar diferenciar y clasificar los diferentes vinagres de vino españoles con DOP mediante su perfil volátil, así como determinar aquellos compuestos volátiles que pueden considerarse marcadores de una DOP o categoría.

Para ello se analizaron por HSSE-GC-MS un total de 50 vinagres de vino de las tres DOPs y de las categorías Crianza, Solera, Reserva y Pedro Ximénez, cuyos cromatograma se procesaron mediante MCR para determinar e identificar los diferentes compuestos volátiles. Los resultados obtenidos fueron sometidos a análisis de varianza (ANOVA), mapas de calor (heatmaps) y análisis discriminante de mínimos cuadrados parciales (PLS-DA).

En este estudio se determinaron por primera vez 7 compuestos volátiles en la DOP Vinagre de Jerez, junto con 28 nuevos compuestos en las otras dos DOPs. Además, las tres DOP de vinagre de vino y sus categorías presentaron diferencias significativas en sus perfiles volátiles, a pesar de mostrar un número total de compuestos similar. Así, se pudieron determinar cómo marcadores de los Vinagres de Condado de Huelva el 1-heptanol, nonanoato de metilo, ácido 2-metilbutanoico, 2,2,6-trimetil-ciclohexanona, trans-2-decenal, eucaliptol y α-terpineol; mientras que diacetilo, acetoína, 3-etoxipropanoato de etilo, 2 y 3-heptanona, 2-metil-1-hexadecanol, 1-octen-3-ol, p-cresol y canfeno fueron seleccionados como marcadores de los Vinagres de Montilla-Moriles y β-damascenona, 5-hidroximetilfurfural, 3-heptanol, trans-2-hexen-1-ol y trans-2-hexen-1-il acetato resultaron ser los marcadores volátiles para la DOP

Vinagre de Jerez. Los modelos de clasificación obtenidos fueron muy satisfactorios ya que fueron capaces de clasificar el 100% de las muestras correctamente, reafirmando la utilidad de los perfiles volátiles para diferenciar, clasificar y autenticar las PDO de vinagre de vino sólo necesitando unos pocos compuestos considerados como marcadores. Estos marcadores de autenticidad podrían contribuir al perfil aromático específico de cada DOP. Por este motivo, para determinar el impacto aromático de estos marcadores en el aroma general de los vinagres de vino DOP, se llevaron a cabo los estudios olfatométricos, los cuales se presentan en el siguiente capítulo (Capítulo VII).

ARTÍCULO 8

Sampling methods for the study of volatile profile of PDO wine vinegars. A comparison using multivariate data analysis

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Sampling methods for the study of volatile profile of PDO wine vinegars. A comparison using multivariate data analysis

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ABSTRACT

High-quality wine vinegars have been registered in Spain under protected designation of origin (PDO): "*Vinagre de Jerez*", "*Vinagre de Condado de Huelva*" and "*Vinagre de Montilla-Moriles*". The raw material, production and aging processes determine their quality and their aromatic composition. Vinegar volatile profile is usually analyzed by gas chromatography–mass spectrometry (GC–MS), being necessary a previous extraction step. Thus, three different sampling methods (Headspace solid phase microextraction "HS-SPME", Headspace stir bar sorptive extraction "HSSE" and Dynamic headspace extraction "DHS") were studied for the analysis of the volatile composition of Spanish PDO wine vinegars. Multivariate curve resolution (MCR) was used to solve chromatographic problems, improving the results obtained. Principal component analysis (PCA) showed that not all the sampling methods were equally suitable for the characterization and differentiation between PDOs and categories, being HSSE the technique that made able the best vinegar characterization.

1. Introduction

Wine vinegar is the most commonly used vinegar in Mediterranean countries and Central Europe (Solieri & Giudici, 2009). It is a product obtained by a double fermentation process (alcoholic and acetous fermentation or acetification). Nowadays, it has become a highly demanded food product, much appreciated in gastronomy, used as a condiment and food preservative (Natera, Castro, De Valme García-Moreno, Hernández, & García-Barroso, 2003). Some wine vinegars are linked to geographical indications and produced within traditional specialties and the European Union has protected them with a legislative system known as "Protected designation of origin" (PDO). This PDO status provides an additional protection of the product against falsifications and it guarantees some specifications related to their chemical and sensory features (Chinnici et al., 2009). In recent years, three high-quality Spanish wine vinegars have been registered under a PDO: "*Vinagre de Jerez*" (also known as "*Sherry wine vinegar*"), "*Vinagre de Condado de Huelva*" and "*Vinagre de Montilla-Moriles*" (*Council Regulation (EC) No 510/2006 of 20 March 2006*). Furthermore, within each PDO, there are different categories according to their aging time and type (static or dynamic) in wood barrels.

The final quality and sensory properties of each wine vinegar are determined by the raw material used, the production process and the aging in wood barrels, with different systems and different aging periods (Callejón, Morales, Silva Ferreira, & Troncoso, 2008; Chinnici et al., 2009). All these variables influence the volatile composition of vinegar and this unique profile represents a fingerprint of each one. For this reason, aroma is considered one of the most important indicators of vinegar quality. Wine vinegar aroma contains a large variety of volatile compounds, with a wide range of polarities, solubility, volatilities and concentrations, making it a complex matrix difficult to qualify and quantify (Blanch, Tabera, Sanz, Herraiz, & Reglero, 1992). Gas chromatography-mass spectrometry (GC–MS) is the most widely employed technique for analyzing the volatile composition, but it is necessary an extraction step prior to GC–MS analysis (Hantao et al., 2012; Marín, Zalacain, De Miguel, Alonso, & Salinas, 2005).

In spite of the fact that different sampling techniques have been applied to extract and concentrate the volatile compounds from wine vinegars, this procedure is still a problem not well resolved due to the wide variety of matrices and the differences in the sampling techniques (Castro Mejías, Natera Marín, De Valme García Moreno, & García Barroso, 2002; Guerrero, Marín, Mejías, & Barroso, 2006). Moreover,

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classical sampling methods, such as solid-phase extraction (SPE) or liquid/liquid extraction, have low reproducibility, high time consumption, and are expensive. For these reasons, there is an increasing trend in the development of new extraction methods with better detection and quantitation limits, that are easy-to-automate, faster and do not consume solvents (Guerrero et al., 2006; Marrufo-Curtido et al., 2012; Pizarro, Esteban-Díez, Sáenz-González, & González-Sáiz, 2008). In general, there are two modes of sampling: direct from the liquid phase or an alternative by the extraction of volatile compounds from the vapor phase above the sample, known as headspace sampling (Morales, Aparicio-Ruiz, & Aparicio, 2013). This mode of sampling has several advantages such as reducing the risk of contamination, the increase of the stir bar and fiber lifetime and a high solute concentration (Bicchi, Iori, Rubiolo, & Sandra, 2002; Weldegergis, Tredoux, & Crouch, 2007). Moreover, regarding the headspace sampling, it can be done in a static (HS) or dynamic (DHS) mode. Headspace solid phase microextraction (HS-SPME) and Headspace Sorptive extraction (HSSE) techniques have been successfully applied in the analysis of solids and liquids (Bicchi et al., 2002; Callejón, González, Troncoso, & Morales, 2008; Weldegergis et al., 2007), whereas DHS method has been less used in vinegar samples (Manzini et al., 2011). Regarding DHS, the main difference with HS is that the sample is purged from the matrix by means of an inert gas and the extracted compounds are trapped and concentrated into a tube filled with a sorbent material. The main advantages of DHS are low detection limits and high sensitivity.

One of the most commonly employed techniques for analyzing the volatile profile of wine vinegars is the Solid phase microextraction (HS-SPME), developed by Arthur and Pawliszyn (1990). It offers important advantages over traditional sampling methods due to its capability of not using extraction solvents, carrying out the extraction and concentration steps simultaneously in a short time (Pizarro et al., 2008). HS-SPME has been widely applied for studying wine (Alves, Nascimento, & Nogueira, 2005; Andujar-Ortiz, Moreno-Arribas, Martín-Alvarez, & Pozo-Bayón, 2009) and vinegar aroma (Aceña, Vera, Guasch, Busto, & Mestres, 2011; Pizarro et al., 2008). The type of polymer on the fiber employed, the extraction time and temperature, the salt concentration and the sample volume are the most important parameters to be considered in HS-SPME methods. However, the lack of sensitivity mainly due to the small amount of polymer is the most important disadvantage of this technique (Baltussen, Cramers, & Sandra, 2002).

Stir bar sorptive extraction (SBSE), a sampling technique developed by Baltussen, Sandra, David, and Cramers (1999), has basic principles of extraction and its advantages are similar to those of HS-SPME. However, this technique has shown higher sensitivity than HS-SPME due to its higher content of extraction polymer employed (Alves et al., 2005; Callejón et al., 2009; Guerrero, Marín, Mejías, & Barroso, 2007). Moreover, the detection limits are improved due to the direct relationship between the amount of analyte extracted and the coating thickness, which is higher in SBSE compared with HS-SPME (David & Sandra, 2007). Successful studies about the application of SBSE in the analysis of the volatile profile in vinegars have been already carried out (Marrufo-Curtido et al., 2012).

Regardless these sampling methods have been widely employed in the analysis of the volatile composition of vinegars (Callejón et al., 2008; Callejón et al., 2008; Casale, Armanino, Casolino, Oliveros, & Forina, 2006; Castro Mejías et al., 2002; Chinnici et al., 2009; Cirlini, Caligiani, Palla, & Palla, 2011; Cocchi, Durante, Marchetti, Armanino, & Casale, 2007; Marrufo-Curtido et al., 2012; Pizarro et al., 2008), the experimental sources of variability related to GC–MS (e.g. columns, stationary phase, temperature or experimental conditions and sample preparation) still cause some variations that directly affects the final results (Amigo et al., 2010). Some of these problems are baseline drifts, co-elution or the presence of unexpected overlapped peaks especially in highly complex samples, decreasing the analytical quality of results (Amigo, Skov, Bro, Coello, & Maspoch, 2008). To handle these problems, some internal standards have been traditionally used. However, they have shown, in many cases, a limited capability of solving severe problems (Oliver-Pozo, Aparicio-Ruiz, Romero, & García-González, 2015). The development of chemometric tools has opened a new way for solving such chromatographic problems and for improving the interpretation of complex data by means of a quick and accurate analysis (Amigo, Skov, & Bro, 2010). In particular, multivariate curve resolution (MCR) is one of the powerful chemometric technique that has increased its application in the resolution of chromatographic and spectral profiles of a wide variety of complex mixtures improving the final chromatographic results (Asadollahi-Baboli & Aghakhani, 2015; Azimi & Fatemi, 2016; Jalali-Heravi, Parastar, & Sereshti, 2008; Zeng, Xu, & Chen, 2009). MCR is a conjunction of algorithms, which helps resolving complex data, transforming it into a simple model of pure responses with a single term per component contribution. It is performed in a mathematical environment, such as MATLAB (Mathworks, Natick, MaA, USA), and can be extended to the analysis of many types of experimental data such as GC–MS data, shortening the time of data processing and improving the results obtained (Hantao et al., 2012).

With all this background, the aim of this study was threefold: firstly, to assess and compare the applicability in the analysis of the volatile composition of Spanish PDO wine vinegars of three different advantageous sampling techniques (HS-SPME-GC–MS, HSSE-GC–MS and DHS-GC–MS). The second one was to demonstrate that the combination of GC–MS analysis and the application of MCR tool was a powerful methodology to overcome common chromatographic problems, to help the extraction of relevant information and identify new analytes in a complex matrix dataset, and to speed up data processing. The third objective was the selection of the best sampling methodology for the characterization and differentiation of PDO wine vinegars and their commercialized categories.

2. Materials and methods

2.1. Samples

Ten wine vinegar samples belonging to the three Spanish PDOs ("*Vinagre de Jerez*", "*Vinagre de Condado de Huelva*" and "*Vinagre de Montilla-Moriles*") were collected from different wineries working in compliance with the Regulatory Councils. Furthermore, two categories were considered in each PDO: "Reserva" wine vinegars (named JRE, CRE and MRE, respectively) aged from more than two years, and the "Pedro Ximenez" category, a sweet category only included in "*Vinagre de Jerez*" and "*Vinagre de Montilla-Moriles*" PDOs (JPX and MPX, respectively). Each sample was analyzed in duplicate by the three sampling methods applied.

2.2. Chemicals and materials

A solution of 4-methyl-2-pentanol (Merck) was employed as internal standard (IS) (6.6 μL in 1 mL of Ethanol flushed with Milli-Q water to a final volume of 10 mL). Sodium chloride and ethanol were of analytical quality and supplied by Merck. Water was obtained from a Milli-Q purification system (Millipore, USA). The retention index (RI) was calculated via injection of a series of C10 to C40 straight-chain n-alkanes (50 mg/L in n-hexane) purchased from Fluka (Madrid, Spain). The volatile compound standards used for the identification of target compounds were purchased from the commercial sources Sigma–Aldrich (Madrid, Spain), Merck (Darmstadt, Germany), and Fluka (Madrid, Spain). Twisters® (10 mm long stir bar coated with 0.5 mm PDMS layer) and Tenax TA™ sorbent tubes used for HSSE and DHS methods respectively, were purchased from Gerstel Inc. (Gerstel, Müllheim and der Ruhr, Germany). The fiber used for HS-SPME method was purchased from Sigma-Aldrich (Bellefonte, PA, USA) and coated with 50/30 μm of divinylbenzene/Carboxen on PDMS fiber (DVB/Carboxen/PDMS).

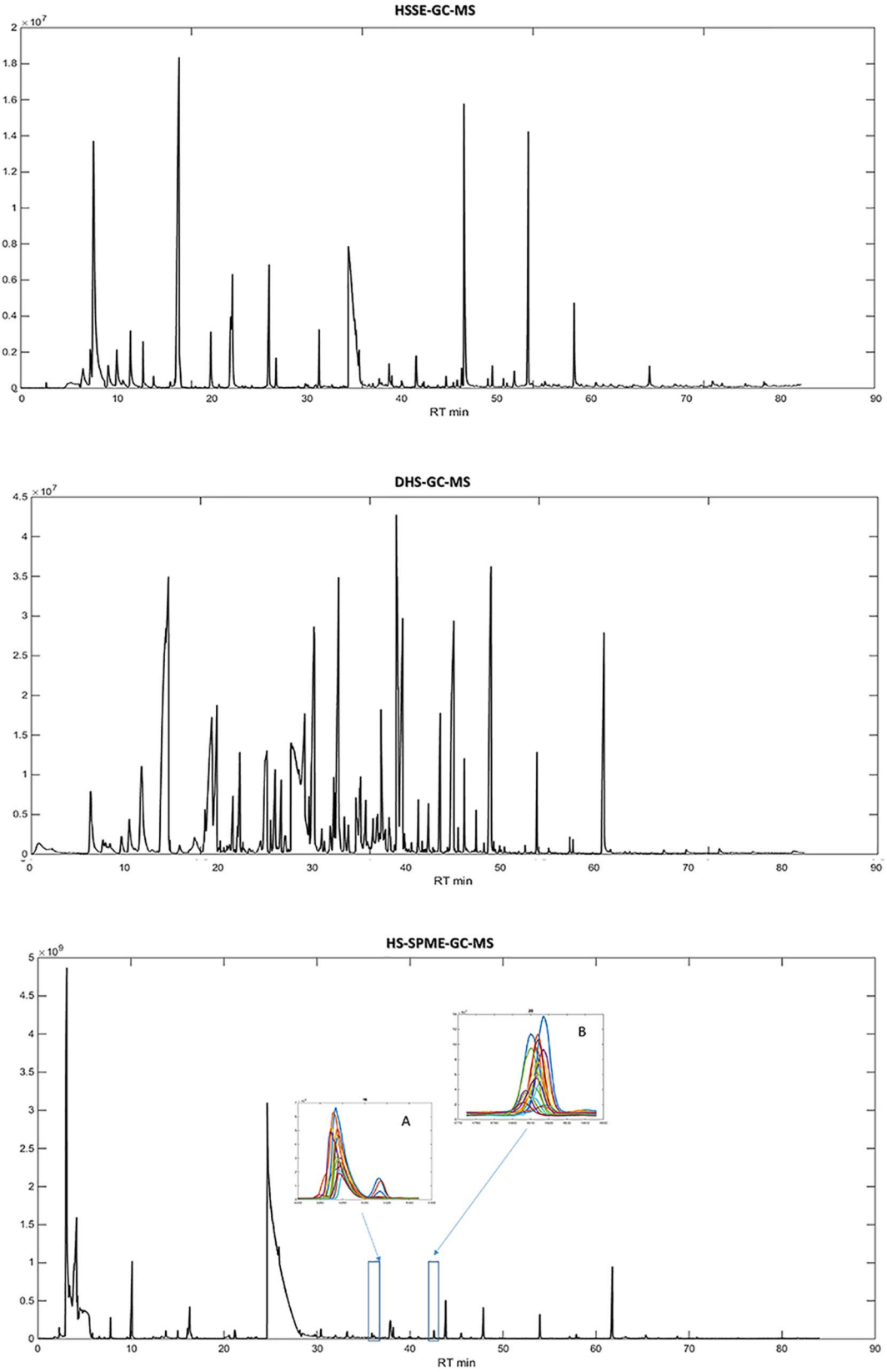

Fig. 1. Example of the total ion chromatogram (TIC) of the PDO wine vinegar analyzed by each sampling method (scales are not equal for a better visualization of chromatograms). Two peak cluster were selected and showed as examples in HS-SPME sampling TIC (A) (B).

2.3. Sampling methods

Three different sampling methods coupled with GC–MS were used for determining the volatile profile of the PDO wine vinegars: HSSE, HS-SPME and DHS. The basic conditions of sample extraction were based on previously reported validated methods (Bicchi et al., 2002; Callejón et al., 2008; Callejón et al., 2008; Natera Marın, Castro Mejıas, de Valme Garcıa Moreno, Garcıa Rowe, & Garcıa Barroso, 2002; Pizarro

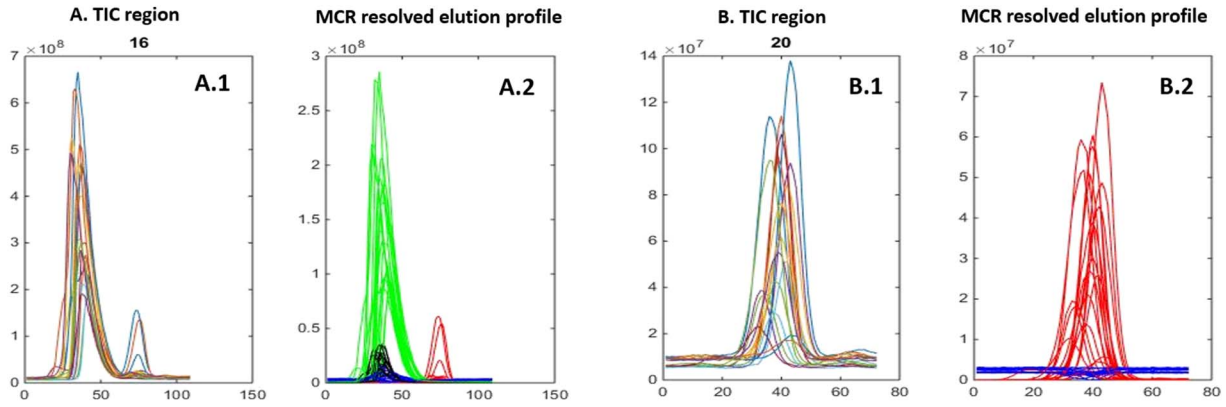

Fig. 2. MCR-resolved elution profiles of two peak clusters (A-B) selected from HS-SPME sampling TIC. (A) Peak cluster and MCR-resolved elution profile example of a completely overlapping components. (B) Peak cluster and MCR-resolved elution profile example of segments with a single component peak.

et al., 2008; Ubeda et al., 2016), which were slightly adapted in order to achieve similar conditions between them that facilitates their comparison. Thus, the raw validated and published conditions for the analysis of volatile compounds in vinegar (i.e. salt content and internal standard) were in certain cases adapted for using a sample volume of 5 mL in the three extraction methods. The samples were analyzed in duplicate and two blank runs were included in the sequence of analysis.

2.3.1. HSSE sampling

Optimal sample extraction and desorption conditions for HSSE were used according to the validated method of Callejón et al. (2008) and Callejón et al. (2008). The desorption step was performed by a Gerstel Thermo Desorption System (TDS2) connected to a cryo-focusing CIS-4 PTV injector (Gerstel).

2.3.2. DHS sampling

The extraction of the volatile fraction of the wine vinegars was carried out by using the Gerstel Dynamic Headspace unit and under sampling conditions from Ubeda et al. (2016). The desorption step was carried out using the same TDS2 and CIS-4 PTV mentioned above.

2.3.3. HS-SPME sampling

The HS-SPME method used for the extraction was adapted from previous validated methods (Cirlini et al., 2011; Natera Marın et al., 2002; Pizarro et al., 2008). Samples were incubated at an agitation temperature of 62 °C. The volatile extraction was carried out by exposing the fiber to the sample headspace (22 mm of fiber depth) during 60 min and agitated at 250 rpm. For desorption of compounds, the fiber was inserted and keep into the injector for 10 min at 240 °C in splitless mode and then at 220 °C for 2 min with a flow rate of 90 mL/min. It was previously conditioned by inserting it into the GC–MS injector at 270 °C for 60 min.

2.3.4. Gas chromatography–mass spectrometry analysis

Two GC–MS systems were used since it was not available an equipment with the three extraction or injection systems (DHS, HSSE and SPME): a 6890 Agilent GC system coupled to a simple quadrupole mass spectrometer Agilent 5975inert for carrying out HSSE and DHS Gerstel extraction and a Bruker 450 GC system coupled to a Bruker 320 triple quadrupole mass spectrometer for HS-SPME method. Analysis were performed in the same conditions and to ensure that the results were comparable, firstly, a comparison between both equipment by the injection of a mixture of standards was performed. Once this test was done this study was carried out. Moreover, we used an internal standard in order to correct the possible variation in sensibility between detectors. Hence, all sampling methods used the same analytical column, a CPWax-57CB column (50 m × 0.25 mm, 0.20-μm film thickness, Varian, Middelburg, Holland) as well as the same oven program. The carrier gas was He at a flow rate of 1 mL/min. The oven temperature program was 35 °C for 5 min, and then raised to 220 °C at 2.5 °C/min (held 5 min). The temperature of transfer line was kept constant at 300 °C. The quadrupole, source and transfer line temperatures were maintained at 150, 230 and 280 °C, respectively. Electron ionization mass spectra in the full-scan mode were recorded at 70 eV electron energy in the range 35–350 *m/z*. All data were recorded using a MS ChemStation version E.02.02.1431 (Agilent technologies Inc.) for HSSE and DHS, and the Star Chromatography Workstation version 6.41 (Varian CA 94598–1675/USA) for HS-SPME.

2.4. Chemometric analysis

2.4.1. Multivariate curve resolution (MCR)

MCR was applied in this study in order to extract the pure mass spectrum and the chromatographic profile of each compound from the original GC–MS data matrix. MCR is a powerful methodology for multiset (multi-sample) analysis. The theory of MCR is fully described in details in the literature (Azimi & Fatemi, 2016; De Juan, Jaumot, & Tauler, 2014; Hantao et al., 2012; Rutan, De Juan, & Tauler, 2009). The first step was to export all the GC–MS files in three-dimensional CSV format for processing in MATLAB version 2014b (The Mathworks Inc., Natick, MA) where all data treatments were performed. All algorithms of chemometric curve resolution were carried out using the PLS_Toolbox 7.9.5 (Eigenvector Research Inc., Wenatchee, WA) working under MATLAB environment and the library searches and spectral matching of the resolved pure components were conducted on the NIST MS database (NIST/EPA/NIH Mass Spectral Library, NIST Scientific and Technical Databases, Gaithersburg, MD 20899–8380). The details of each developed step is presented as follows:

(1) Data processing: The final data form was a three-way array, **X** (I x J x K), in which the three modes accounted for elution time (I scans), spectral domain (*m/z* fragments "J") and samples (K), respectively. In the present work, the three-way array **X** was 22,845 × 316 × 20 (10 samples in duplicate). Once the data was structured in the correct way, the total ion chromatogram (TIC) for each sample was overlaid. Then, for increasing the efficiency of MCR in terms of computation time (Amigo et al., 2010), the entire chromatographic dataset was inspected and divided into different intervals and each part was analyzed by independent MCR models. The intervals were selected accordingly to previous knowledge about the GC–MS analysis of some wine vinegars (Callejón et al., 2008; Callejón et al., 2008; Callejón, Torija, Mas, Morales, & Troncoso, 2010). The nonnegativity constraints were incorporated on the conditionally linear parameters of the chromatographic and spectral profiles.
(2) Determination of the correct number of chemical factors: There are many methods for chemical data rank determination such as

Table 1
Mean relative area of the volatile compounds extracted in the different wine vinegar samples by each sampling method.

				CRE						JRE						MRE	
Nº	Volatile compounds[a]	ID[b]	RI[c]	HSSE		DHS		HSSPME		HSSE		DHS		HSSPME		HSSE	
				ARm[d]	± SD	ARm	± SD	ARm	± SD	ARm	± SD	ARm	± SD	ARm	± SD	ARm	± SD
Acetic esters																	
1	Ethyl acetate	A	903	48.2	8.16	25.4	21.1	nd	–	42.5	0.61	15.7	7.90	nd	–	29.4	21.5
2	n-Propyl acetate	A	947	1.47	1.31	1.55	1.76	nd	–	0.88	0.67	0.85	0.59	nd	–	0.17	0.20
3	sec-Butyl acetate	A	959	0.28	0.28	nd	–	nd	–	0.09	0.03	nd	–	nd	–	0.16	0.21
4	Isobutyl acetate[a]	A	983	6.46	4.60	3.66	2.98	0.51	0.15	4.45	2.12	2.48	1.02	0.71	0.54	2.00	0.20
5	2-Methylbutyl acetate[a]	A	1091	8.69	6.46	9.82	8.57	2.37	1.02	5.59	0.60	6.36	1.05	2.19	1.06	3.36	0.25
6	Isoamyl acetate[a]	A	1091	40.1	35.8	73.6	79.6	6.37	4.52	21.2	5.34	33.7	11.6	4.53	3.16	10.3	3.35
7	Hexyl acetate	A	1253	0.54	0.72	1.82	2.51	nd	–	0.23	0.13	0.34	0.09	nd	–	0.09	0.12
8	Butyl acetate	B[54,56]	1044	nd	–	1.81	2.12	nd	–	nd	–	0.60	0.27	nd	–	nd	–
9	3-Ethoxypropyl acetate	C	1348	0.07	0.03	1.76	2.00	nd	–	0.07	0.03	1.04	0.89	nd	–	0.10	0.04
10	2,3-Butanediol diacetate	B[54]	1477	0.14	0.04	1.05	1.12	nd	–	0.17	0.08	1.25	0.91	nd	–	0.28	0.14
11	1,3-Propanediol, diacetate[a]	B[54]	1654	0.14	0.01	3.81	3.60	0.23	0.02	0.10	0.06	1.18	0.86	0.12	0.05	0.22	0.20
12	2,3-Dihydroxypro-pyl acetate	B[54]	2282	nd	–	0.51	0.43	0.34	0.04	nd	–	0.19	0.10	0.02	0.00	nd	–
13	Benzyl acetate[a]	A	1720	0.12	0.05	1.22	1.36	0.10	0.02	0.20	0.10	1.25	0.85	0.16	0.07	0.12	0.07
14	2-Phenylethyl acetate[a]	A	1810	5.19	2.55	22.2	25.2	4.78	1.12	6.28	2.67	15.8	9.42	5.73	2.14	5.92	1.77
15	Methyl acetate	B[54]	874	1.04	0.11	nd	–	nd	–	0.92	0.21	nd	–	nd	–	0.97	0.08
Total of acetic esters				112	60.2	148	152	14.7	6.90	82.7	12.6	80.9	35.6	13.5	7.03	53.1	28.1
Acids																	
16	Acetic acid[a]	A	1437	9.27	0.00	40.1	9.85	3.26	0.62	10.4	7.89	55.4	37.7	4.24	3.54	14.02	1.25
17	Propanoic acid	A	1518	nd	–	2.19	1.37	0.37	0.09	nd	–	2.65	0.80	0.27	0.10	nd	–
18	Isobutyric acid[a]	A	1564	0.32	0.04	3.02	0.94	0.56	0.08	0.39	0.31	5.82	3.01	0.58	0.29	0.35	0.01
19	Butanoic acid[a]	A	1629	0.11	0.03	10.30	7.86	0.53	0.08	0.16	0.19	13.9	12.4	0.52	0.40	0.21	0.20
20	Isovaleric acid[a]	A	1669	2.93	0.59	24.4	16.7	3.95	0.52	3.84	2.03	27.6	6.79	4.87	0.94	4.64	0.70
21	2-methylbutanoic acid	A	1539	nd	–	nd	–	0.48	0.04	nd	–	nd	–	0.57	0.26	nd	–
22	Pentanoic acid	A	1722	nd	–	0.21	0.20	0.08	0.01	nd	–	0.34	0.17	0.13	0.05	nd	–
23	Hexanoic acid[a]	A	1855	0.07	0.01	3.28	3.42	0.73	0.17	0.11	0.08	3.84	1.02	0.71	0.18	0.12	0.01
24	2-Ethylhexanoic acid[a]	B[1]	1958	0.02	0.01	0.21	0.23	0.08	0.02	0.07	0.01	0.27	0.21	0.12	0.06	0.03	0.01
25	Octanoic acid[a]	A	2084	0.12	0.03	3.38	3.57	1.87	0.28	0.17	0.12	3.07	1.20	1.99	0.46	0.11	0.03
26	Sorbic Acid	B[1,55]	2138	nd	–	0.03	0.04	0.20	0.09	nd	–	0.12	0.17	0.04	0.02	nd	–
27	Nonanoic acid	A	2163	nd	–	0.30	0.32	0.40	0.11	nd	–	0.22	0.10	0.46	0.06	nd	–
28	Decanoic acid[a]	A	2298	0.19	0.03	10.7	10.2	5.28	1.53	0.15	0.12	7.38	6.03	4.89	0.99	0.18	0.09
29	Dodecanoic acid	B[1]	2456	nd	–	0.17	0.17	0.33	0.12	nd	–	0.24	0.31	0.26	0.12	nd	–
30	Tetradecanoic acid	B[1,2]	2[6]0	nd	–	0.22	0.22	nd	–	nd	–	0.28	0.30	nd	–	nd	–
31	Pentadecanoic acid	B[1,2]	2670	nd	–	0.08	0.09	nd	–	nd	–	0.13	0.13	nd	–	nd	–
32	Hexadecanoic acid	B[1,2]	2776	nd	–	0.30	0.28	nd	–	nd	–	0.51	0.33	nd	–	nd	–
Total of acids				13.04	0.75	98.9	[2].6	18.1	3.70	15.3	10.7	121	70.6	19.6	7.5	19.6	2.31
Alcohols																	
33	Ethanol	A	932	5.88	4.62	nd	–	nd	–	5.79	2.42	nd	–	nd	–	1.36	1.24
34	Isoamyl alcohol	A	1207	7.90	8.08	21.7	23.6	nd	–	4.48	1.99	8.00	3.[1]	nd	–	2.14	0.66
35	2-Methy-1-butanol[a]	A	1207	4.77	2.98	7.44	6.19	1.83	0.97	3.38	0.96	4.62	1.02	1.42	0.[4]	2.06	0.09
36	2-Ethyl-1-hexanol	A	1489	0.12	0.05	nd	–	nd	–	0.16	0.01	nd	–	nd	–	0.08	0.04
37	2-Phenylethanol[a]	A	1930	1.[1]	0.[5]	33.3	37.4	3.77	1.34	1.85	0.63	20.1	9.80	3.39	1.07	1.60	0.34
38	1-Hexanol	A	1348	nd	–	5.71	7.84	nd	–	nd	–	1.36	0.38	nd	–	nd	–
39	1,3-Propanediol	B[1]	18[6]	nd	–	0.09	0.06	nd	–	nd	–	0.13	0.11	nd	–	nd	–
40	2-Furanmethanol	A	1669	0.41	0.14	nd	–	nd	–	0.46	0.07	nd	–	nd	–	0.29	0.13

(continued on next page)

Table 1 *(continued)*

Nº	Volatile compounds[a]	ID[b]	RI[c]	CRE						JRE						MRE	
				HSSE		DHS		HSSPME		HSSE		DHS		HSSPME		HSSE	
				ARm[d]	± SD	ARm	± SD	ARm	± SD	ARm	± SD	ARm	± SD	ARm	± SD	ARm	± SD
Total of alcohols				20.6	16.4	68.2	75.0	5.61	2.31	16.1	6.06	34.2	14.8	4.81	1.65	7.53	2.50
Aldehydes																	
41	Benzaldehyde[a]	A	1514	0.87	1.13	21.0	28.8	0.51	0.[5]	0.47	0.02	5.81	0.96	0.36	0.08	1.17	1.17
42	Furfural	A	1437	1.14	0.79	32.6	39.7	nd	–	1.53	0.89	29.6	12.9	nd	–	1.00	0.81
43	5-Methylfurfural[a]	A	1[3]4	0.08	0.05	1.08	1.33	0.04	0.04	0.11	0.09	1.05	0.62	0.07	0.03	0.06	0.06
44	5-Acetoxymethyl-2-furaldehyde	B[1]	2191	nd	–	0.02	0.02	nd	–	nd	–	0.04	0.03	nd	–	nd	–
45	5-Hydroxymethylfurfural	A	2480	nd	–	0.06	0.03	0.10	0.03	nd	–	0.10	0.00	0.19	0.01	nd	–
Total of aldehydes				2.09	1.97	[1].78	69.83	0.66	0.65	2.12	1.00	36.6	14.5	0.62	0.12	2.23	2.04
Ethyl esters																	
46	Ethyl propanoate	A	932	1.[3]	1.42	1.69	2.02	nd	–	0.70	0.43	0.[2]	0.32	nd	–	0.29	0.38
47	Ethyl isobutyrate	A	947	1.89	1.64	0.78	0.75	nd	–	1.11	0.64	0.45	0.18	nd	–	0.18	0.21
48	Ethyl butyrate[a]	A	999	1.50	1.34	4.[5]	5.33	0.14	0.07	0.88	0.90	1.66	1.[3]	0.18	0.21	0.32	0.45
49	Ethyl 2-methylbutyrat[a]	A	1014	1.16	0.94	0.[1]	0.44	0.09	0.03	0.61	0.22	0.26	0.11	0.10	0.08	0.10	0.11
50	Ethyl isovalerate[a]	A	1036	8.89	6.96	6.21	5.04	1.05	0.47	6.19	0.53	4.75	0.60	1.15	0.[6]	0.91	1.10
51	Ethyl pentanoate	A	1093	nd	–	0.53	0.62	nd	–	nd	–	0.22	0.11	nd	–	nd	–
52	Ethyl hexanoate[a]	A	1207	1.[4]	1.84	9.12	12.33	0.34	0.33	0.52	0.15	1.32	0.86	0.16	0.11	0.10	0.11
53	Ethyl 3-ethoxypropanoate	B[1,2]	1326	nd	–	0.80	1.05	nd	–	nd	–	0.24	0.18	nd	–	nd	–
[1]	Ethyl heptanoate	A	1319	0.04	0.05	nd	–	nd	–	0.01	0.01	nd	–	nd	–	0.00	0.00
[2]	Ethyl octanoate	A	1437	1.47	1.91	13.52	18.40	nd	–	0.44	0.01	1.[5]	0.66	nd	–	0.06	0.06
[3]	Ethyl sorbate	B[1]	1491	0.21	0.23	2.23	3.07	nd	–	0.04	0.01	0.04	0.01	nd	–	0.04	0.03
[4]	Ethyl decanoate	A	1629	0.22	0.28	nd	–	0.23	0.19	0.07	0.04	nd	–	0.12	0.01	0.01	0.01
[5]	Ethyl benzoate	A	16[1]	0.13	0.10	nd	–	0.09	0.04	0.07	0.03	nd	–	0.06	0.03	0.04	0.03
[6]	Diethyl succinate[a]	A	1669	0.81	0.99	24.3	33.9	0.71	0.85	0.32	0.34	2.42	2.78	0.22	0.22	0.07	0.04
60	Ethyl phenyl acetate[a]	A	1779	1.18	0.84	11.02	13.33	1.02	0.43	1.19	0.79	6.11	4.81	1.02	0.[6]	0.20	0.21
61	Ethyl dodecanoate	A	1818	0.01	0.00	0.05	0.07	nd	–	0.01	0.00	0.01	0.00	nd	–	0.01	0.01
62	Diethyl malate	B[2]	2038	nd	–	0.07	0.10	nd	–	nd	–	0.01	0.01	nd	–	nd	–
63	Methyl hexadecanoate	B[1]	2222	0.05	0.02	nd	–	nd	–	0.09	0.01	nd	–	nd	–	0.07	0.06
64	Ethyl hexadecanoate	A	2260	0.02	0.01	nd	–	nd	–	0.01	0.01	nd	–	nd	–	0.01	0.00
65	Ethyl furoate[a]	A	1608	0.10	0.05	1.41	1.63	0.05	0.01	0.08	0.03	0.74	0.40	0.05	0.02	0.03	0.01
66	Ethyl hydrogen succinate	B[4]	22[1]	nd	–	nd	–	0.21	0.20	nd	–	nd	–	0.11	0.08	nd	–
Total of ethyl esters				20.8	18.6	76.8	98.1	3.92	2.63	12.3	4.15	20.4	12.6	3.17	1.94	2.43	2.83
Ketones																	
67	2,3-Butanedione	C	947	1.[1]	1.26	nd	–	nd	–	1.23	0.27	nd	–	nd	–	2.[6]	0.76
68	Methyl Isobutyl ketone	B[1,2]	983	0.20	0.16	nd	–	nd	–	0.37	0.38	nd	–	nd	–	0.04	0.01
69	Acetoin	A	1275	0.[5]	0.39	5.27	4.80	nd	–	0.90	0.41	4.16	2.01	nd	–	2.09	1.61
70	1-Hydroxy-2-propanone	A	1279	1.50	0.35	nd	–	nd	–	1.[3]	0.15	nd	–	nd	–	1.[5]	1.04
71	3-Nonanone	A	1348	0.06	0.06	nd	–	nd	–	0.02	0.01	nd	–	nd	–	0.01	0.01
72	2-Acetoxy-3-butanone	B[1]	1370	0.14	0.06	nd	–	nd	–	0.23	0.14	nd	–	nd	–	0.[2]	0.30
73	2-Acethylfuran	A	1498	0.10	0.04	1.60	1.71	nd	–	0.12	0.04	1.70	0.21	nd	–	0.09	0.04
74	2(5H)-Furanone	B[5]	17[4]	0.23	0.07	nd	–	nd	–	0.26	0.03	nd	–	nd	–	0.19	0.08
Total of ketones				4.33	2.40	6.87	6.51	0.00	0.00	4.69	1.45	5.86	2.22	0.00	0.00	7.13	3.85
Others																	
75	Trans-linalool oxide	B[6]	1460	0.09	0.04	nd	–	nd	–	0.08	0.02	nd	–	nd	–	0.05	0.02
76	TDN[a]	B[2]	1732	0.17	0.14	0.35	0.25	0.13	0.01	0.09	0.03	0.12	0.07	0.06	0.01	0.13	0.17
77	2,4,5-Trimethyl-1,3-Dioxolane	B[2]	932	2.82	2.41	2.38	2.03	nd	–	1.04	0.63	0.62	0.33	nd	–	0.77	0.51
78	1,4:3,6-Dianhydro-α-d-glucopyranose	C	2433	0.06	0.01	0.02	0.02	nd	–	0.08	0.04	0.05	0.05	nd	–	0.05	0.00
79	Methyl salicylate[a]	A	1765	0.02	0.00	0.11	0.11	0.02	0.00	0.08	0.04	0.41	0.29	0.08	0.05	0.02	0.01

(continued on next page)

Table 1 *(continued)*

Nº	Volatile compounds[a]	ID[b]	RI[c]	CRE						JRE						MRE	
				HSSE		DHS		HSSPME		HSSE		DHS		HSSPME		HSSE	
				ARm[d]	± SD	ARm	± SD	ARm	± SD	ARm	± SD	ARm	± SD	ARm	± SD	ARm	± SD
80	Isopropyl myristate	B[1]	2041	0.02	0.01	nd	–	nd	–	0.02	0.01	nd	–	nd	–	0.07	0.04
81	4-Ethylguaiacol	A	2041	0.03	0.01	0.25	0.27	nd	–	0.07	0.01	0.36	0.09	nd	–	0.03	0.01
Total of others				3.20	2.61	3.11	2.68	0.15	0.01	1.47	0.77	1.[3]	0.82	0.14	0.05	1.12	0.77

Nº	Volatile compounds[a]	MRE				JPX						MPX					
		DHS		HSSPME		HSSE		DHS		HSSPME		HSSE		DHS		HSSPME	
		ARm	± SD	ARm	± SD	ARm	± SD	ARm	± SD	ARm	± SD	ARm	± SD	ARm	± SD	ARm	± SD
Acetic esters																	
1	Ethyl acetate	2.95	3.70	nd	–	54.7	17.9	20.7	0.90	nd	–	39.0	0.72	8.68	2.90	nd	–
2	n-Propyl acetate	0.21	0.24	nd	–	1.34	0.02	1.17	0.14	nd	–	0.13	0.01	0.26	0.24	nd	–
3	sec-Butyl acetate	nd	–	nd	–	0.07	0.05	nd	–	nd	–	0.03	0.00	nd	–	nd	–
4	Isobutyl acetate[a]	0.97	0.27	0.22	0.00	6.63	0.96	3.30	0.13	2.36	1.78	1.57	0.02	1.25	0.56	1.03	0.22
5	2-Methylbutyl acetate[a]	3.32	0.42	1.05	0.09	6.28	0.38	6.66	0.62	6.55	5.10	2.46	0.03	2.91	0.74	4.99	0.08
6	Isoamyl acetate[a]	14.8	5.99	1.63	0.41	29.9	6.94	41.9	0.12	19.8	18.49	8.44	0.13	13.3	9.05	8.81	4.64
7	Hexyl acetate	0.12	0.15	nd	–	0.16	0.03	0.25	0.04	nd	–	0.03	0.00	0.04	0.01	nd	–
8	Butyl acetate	0.40	0.42	nd	–	nd	–	0.78	0.02	nd	–	nd	–	0.19	0.19	nd	–
9	3-Ethoxypropyl acetate	0.96	0.56	nd	–	0.09	0.00	1.62	0.08	nd	–	0.03	0.00	0.46	0.46	nd	–
10	2,3-Butanediol diacetate	1.62	0.94	nd	–	0.19	0.04	1.38	0.74	nd	–	0.11	0.01	0.46	0.53	nd	–
11	1,3-Propanediol, diacetate[a]	1.79	1.61	0.21	0.15	0.11	0.06	1.49	0.43	0.30	0.21	0.04	0.01	0.56	0.63	0.29	0.12
12	2,3-Dihydroxypro-pyl acetate	0.24	0.06	0.78	0.20	nd	–	0.27	0.01	0.12	0.13	nd	–	0.08	0.03	0.06	0.01
13	Benzyl acetate[a]	0.64	0.42	0.12	0.05	0.19	0.11	1.31	0.76	0.37	0.23	0.18	0.02	0.90	1.17	0.96	1.17
14	2-Phenylethyl acetate[a]	12.6	4.95	5.60	0.57	6.74	2.02	18.3	5.92	16.3	12.7	2.63	0.22	4.98	3.36	14.6	5.51
15	Methyl acetate	nd	–	nd	–	1.18	0.16	nd	–	nd	–	0.55	0.00	nd	–	nd	–
Total of acetic esters		40.7	19.7	9.60	1.47	107	28.7	99.1	9.91	45.8	38.7	55.2	1.18	34.0	19.9	30.75	11.7
Acids																	
16	Acetic acid[a]	25.7	9.38	4.94	4.42	14.1	2.63	26.2	13.2	12.3	7.16	8.90	0.32	20.7	2.79	9.51	6.38
17	Propanoic acid	3.12	2.39	0.32	0.16	nd	–	2.28	1.32	0.59	0.35	nd	–	1.09	0.81	0.65	0.30
18	Isobutyric acid[a]	4.32	1.19	0.40	0.02	0.43	0.26	5.56	3.37	1.03	0.34	0.16	0.00	2.08	0.80	0.81	0.13
19	Butanoic acid[a]	11.5	12.7	0.55	0.42	0.19	0.15	13.2	13.5	1.25	0.63	0.05	0.00	1.91	0.92	1.08	0.04
20	Isovaleric acid[a]	18.9	5.14	5.01	0.99	4.04	1.75	26.4	8.53	10.1	6.43	2.60	0.01	13.27	4.66	14.6	3.38
21	2-methylbutanoic acid	nd	–	0.45	0.08	nd	–	nd	–	0.99	0.34	nd	–	nd	–	0.92	0.21
22	Pentanoic acid	0.26	0.22	0.08	0.01	nd	–	0.33	0.19	0.31	0.22	nd	–	0.09	0.05	0.27	0.03
23	Hexanoic acid[a]	2.49	0.73	0.64	0.08	0.14	0.04	3.43	1.60	1.92	1.53	0.08	0.00	1.22	0.67	1.97	0.56
24	2-Ethylhexanoic acid[a]	0.11	0.06	0.06	0.01	0.05	0.04	0.31	0.16	0.25	0.13	0.01	0.00	0.04	0.01	0.15	0.01
25	Octanoic acid[a]	2.21	1.13	2.26	0.27	0.15	0.14	2.21	0.02	4.03	2.44	0.07	0.00	1.51	0.88	6.49	1.13
26	Sorbic Acid	0.04	0.05	0.09	0.02	nd	–	0.00	0.00	0.09	0.04	nd	–	0.03	0.04	0.11	0.07
27	Nonanoic acid	0.16	0.07	0.55	0.03	nd	–	0.16	0.01	1.39	1.25	nd	–	0.12	0.05	2.23	0.22
28	Decanoic acid[a]	5.75	2.54	5.17	1.12	0.14	0.13	4.00	1.25	8.75	4.47	0.10	0.02	3.91	1.72	13.1	6.81
29	Dodecanoic acid	0.13	0.08	0.32	0.11	nd	–	0.03	0.00	0.71	0.51	nd	–	0.07	0.06	2.83	2.27
30	Tetradecanoic acid	0.18	0.10	nd	–	nd	–	0.05	0.02	nd	–	nd	–	0.08	0.08	nd	–
31	Pentadecanoic acid	0.07	0.04	nd	–	nd	–	0.03	0.02	nd	–	nd	–	0.03	0.03	nd	–
32	Hexadecanoic acid	0.22	0.11	nd	–	nd	–	0.20	0.11	nd	–	nd	–	0.12	0.08	nd	–
Total of acids		75.3	35.9	20.8	7.73	19.2	5.14	84.4	43.3	43.7	25.8	11.9	0.36	46.3	13.6	[1].7	21.5
Alcohols																	
33	Ethanol	nd	–	nd	–	6.81	0.97	nd	–	nd	–	3.28	0.14	nd	–	nd	–

(continued on next page)

Table 1 *(continued)*

Nº	Volatile compounds[a]	MRE				JPX						MPX					
		DHS		HSSPME		HSSE		DHS		HSSPME		HSSE		DHS		HSSPME	
		ARm	± SD	ARm	± SD	ARm	± SD	ARm	± SD	ARm	± SD	ARm	± SD	ARm	± SD	ARm	± SD
34	Isoamyl alcohol	3.47	1.70	nd	–	5.67	0.30	10.6	0.15	nd	–	2.[4]	0.07	5.00	4.68	nd	–
35	2-Methy-1-butanol[a]	2.[1]	0.50	0.74	0.00	3.[2]	0.72	4.85	0.70	3.49	2.36	2.14	0.06	3.00	1.68	3.93	1.65
36	2-Ethyl-1-hexanol	nd	–	nd	–	0.15	0.04	nd	–	nd	–	0.14	0.00	nd	–	nd	–
37	2-Phenylethanol[a]	15.0	5.19	3.[3]	0.01	1.76	0.75	22.1	6.94	10.2	8.50	1.17	0.20	10.8	8.80	15.0	10.4
38	1-Hexanol	0.[2]	0.71	nd	–	nd	–	0.93	0.22	nd	–	nd	–	0.19	0.09	nd	–
39	1,3-Propanediol	0.04	0.01	nd	–	nd	–	0.14	0.09	nd	–	nd	–	0.04	0.01	nd	–
40	2-Furanmethanol	nd	–	nd	–	0.38	0.06	nd	–	nd	–	0.25	0.07	nd	–	nd	–
Total of alcohols		21.6	8.11	4.30	0.02	18.3	2.83	38.6	8.10	13.6	10.9	9.[2]	0.53	19.0	15.3	18.9	12.0
Aldehydes																	
41	Benzaldehyde[a]	10.8	11.2	0.68	0.[2]	0.29	0.23	3.71	3.92	0.45	0.06	0.22	0.00	1.61	0.66	0.93	0.01
42	Furfural	16.6	17.4	nd	–	0.75	0.21	14.2	8.90	nd	–	0.80	0.05	10.4	7.52	nd	–
43	5-Methylfurfural[a]	0.53	0.62	0.03	0.03	0.06	0.01	0.[5]	0.05	0.10	0.08	0.10	0.00	0.76	0.27	0.37	0.23
44	5-Acetoxymethyl-2-furaldehyde	0.01	0.01	nd	–	nd	–	0.04	0.03	nd	–	nd	–	0.07	0.02	nd	–
45	5-Hydroxymethylfurfural	0.02	0.01	0.06	0.01	nd	–	0.14	0.05	0.50	0.42	nd	–	0.14	0.06	1.10	0.04
Total of aldehydes		27.9	29.2	0.76	0.[6]	1.10	0.45	18.6	12.9	1.05	0.[3]	1.13	0.06	12.9	8.53	2.40	0.28
Ethyl esters																	
46	Ethyl propanoate	0.19	0.26	nd	–	0.79	0.29	0.[5]	0.28	nd	–	0.21	0.01	0.28	0.21	nd	–
47	Ethyl isobutyrate	0.10	0.11	nd	–	1.28	0.39	0.43	0.21	nd	–	0.24	0.00	0.15	0.04	nd	–
48	Ethyl butyrate[a]	0.[6]	0.83	0.04	0.06	0.97	0.79	1.67	1.[3]	0.30	0.04	0.09	0.00	0.23	0.09	0.07	0.02
49	Ethyl 2-methylbutyrat[a]	0.04	0.05	0.01	0.01	0.69	0.09	0.30	0.07	0.26	0.15	0.13	0.00	0.07	0.00	0.09	0.04
50	Ethyl isovalerate[a]	0.65	0.81	0.12	0.13	6.40	0.24	4.62	0.79	2.98	2.00	1.80	0.03	1.[5]	0.03	1.65	0.52
51	Ethyl pentanoate	0.08	0.06	nd	–	nd	–	0.27	0.05	nd	–	nd	–	0.07	0.04	nd	–
52	Ethyl hexanoate[a]	0.17	0.20	0.02	0.03	0.72	0.13	1.65	0.39	0.65	0.[6]	0.15	0.00	0.23	0.15	0.24	0.03
53	Ethyl 3-ethoxypropanoate	0.13	0.14	nd	–	nd	–	0.27	0.14	nd	–	nd	–	0.06	0.06	nd	–
[1]	Ethyl heptanoate	nd	–	nd	–	0.02	0.01	nd	–	nd	–	0.00	0.00	nd	–	nd	–
[2]	Ethyl octanoate	0.26	0.24	nd	–	0.44	0.00	1.65	0.[2]	nd	–	0.15	0.00	0.43	0.23	nd	–
[3]	Ethyl sorbate	0.06	0.05	nd	–	0.04	0.00	0.04	0.00	nd	–	0.03	0.00	0.05	0.05	nd	–
[4]	Ethyl decanoate	nd	–	0.01	0.01	0.04	0.01	nd	–	0.17	0.05	0.05	0.00	nd	–	0.23	0.02
[5]	Ethyl benzoate	nd	–	0.03	0.02	0.09	0.01	nd	–	0.21	0.18	0.05	0.00	nd	–	0.25	0.17
[6]	Diethyl succinate[a]	0.44	0.37	0.06	0.03	0.34	0.31	2.69	2.39	0.52	0.21	0.07	0.01	0.61	0.77	0.40	0.41
60	Ethyl phenyl acetate[a]	0.98	1.11	0.24	0.23	1.14	0.87	6.17	4.72	2.13	0.99	0.21	0.02	0.83	0.[2]	1.44	0.36
61	Ethyl dodecanoate	0.00	0.00	nd	–	0.01	0.01	0.01	0.00	nd	–	0.03	0.00	0.02	0.02	nd	–
62	Diethyl malate	0.00	0.00	nd	–	nd	–	0.01	0.00	nd	–	nd	–	0.00	0.00	nd	–
63	Methyl hexadecanoate	nd	–	nd	–	0.05	0.03	nd	–	nd	–	0.06	0.03	nd	–	nd	–
64	Ethyl hexadecanoate	nd	–	nd	–	0.01	0.01	nd	–	nd	–	0.04	0.00	nd	–	nd	–
65	Ethyl furoate[a]	0.23	0.12	0.02	0.01	0.09	0.02	0.86	0.23	0.15	0.12	0.04	0.00	0.25	0.28	0.13	0.11
66	Ethyl hydrogen succinate	nd	–	0.05	0.02	nd	–	nd	–	0.18	0.02	nd	–	nd	–	0.08	0.10
Total of ethyl esters		3.92	4.34	0.61	0.[1]	13.1	3.21	21.2	11.4	7.[4]	4.35	3.37	0.13	4.86	2.52	4.60	1.78
Ketones																	
67	2,3-Butanedione	nd	–	nd	–	1.40	0.02	nd	–	nd	–	0.86	0.01	nd	–	nd	–
68	Methyl Isobutyl ketone	nd	–	nd	–	0.09	0.02	nd	–	nd	–	0.03	0.00	nd	–	nd	–
69	Acetoin	6.61	3.98	nd	–	1.06	0.18	5.68	0.14	nd	–	0.74	0.01	5.25	5.63	nd	–
70	1-Hydroxy-2-propanone	nd	–	nd	–	1.94	0.38	nd	–	nd	–	1.42	0.26	nd	–	nd	–
71	3-Nonanone	nd	–	nd	–	0.02	0.00	nd	–	nd	–	0.01	0.00	nd	–	nd	–
72	2-Acetoxy-3-butanone	nd	–	nd	–	0.29	0.06	nd	–	nd	–	0.20	0.01	nd	–	nd	–
73	2-Acethylfuran	0.78	0.[5]	nd	–	0.11	0.03	1.70	0.20	nd	–	0.12	0.00	1.39	0.62	nd	–

(continued on next page)

Table 1 (*continued*)

		MRE				JPX				MPX					
Nº	Volatile compounds[a]	DHS		HSSPME		HSSE		DHS		HSSPME		HSSE		DHS		HSSPME	
		ARm	± SD	ARm	± SD	ARm	± SD	ARm	± SD	ARm	± SD	ARm	± SD	ARm	± SD	ARm	± SD
74	2(5H)-Furanone	nd	–	nd	–	0.23	0.00	nd	–	nd	–	0.17	0.02	nd	–	nd	–
Total of ketones		7.39	4.[3]	0.00	0.00	5.14	0.70	7.38	0.34	0.00	0.00	3.[2]	0.32	6.64	6.25	0.00	0.00
Others																	
75	Trans-linalool oxide	nd	–	nd	–	0.06	0.01	nd	–	nd	–	0.12	0.00	nd	–	nd	–
76	TDN[a]	0.13	0.15	0.10	0.13	0.06	0.01	0.05	0.02	0.11	0.08	0.70	0.00	0.41	0.[1]	2.32	3.05
77	2,4,5-Trimethyl-1,3-Dioxolane	0.39	0.32	nd	–	1.17	0.44	0.66	0.28	nd	–	0.41	0.04	0.25	0.29	nd	–
78	1,4:3,6-Dianhydro-α-d-glucopyranose	0.01	0.00	nd	–	0.06	0.00	0.07	0.02	nd	–	0.05	0.00	0.01	0.01	nd	–
79	Methyl salicylate[a]	0.07	0.04	0.02	0.01	0.07	0.06	0.37	0.35	0.13	0.02	0.01	0.00	0.03	0.01	0.08	0.01
80	Isopropyl myristate	nd	–	nd	–	0.05	0.02	nd	–	nd	–	0.10	0.08	nd	–	nd	–
81	4-Ethylguaiacol	0.15	0.06	nd	–	0.06	0.02	0.36	0.09	nd	–	0.02	0.00	0.07	0.05	nd	–
Total of others		0.75	0.[4]	0.12	0.13	1.52	0.[4]	1.51	0.76	0.24	0.10	1.41	0.14	0.78	0.89	2.40	3.06

nd: peak not detected.

[a] Volatile compounds extracted in common by the three sampling methods.

[b] ID (identification): reliability of identification: A, mass spectrum and LRI agreed with standards; B, mass spectrum agreed with mass spectral data base and LRI agreed with the literature data obtained with standards; C, mass spectrum agreed with mass spectral data base. Reference which matched the experimental LRI: 1: National Center for Biotechnology Information (2005); 2: Morales et al. (2017); 3: Ruiz-Bejarano et al. (2013); 4: Selli et al. (2004); 5: Pozo-Bayón et al. (2007); 6: Loscos et al. (2007).

[c] RI: Retention Index.

[d] ARm: Mean relative areas.

principal component analysis (PCA), orthogonal projection approach (OPA) and SIMPLISIMA (Azimi & Fatemi, 2016; Jalali-Heravi et al., 2008). However, due to the accumulation of noise in complex system analyzed by GC–MS, it is often difficult to achieve a true rank. In the present study, and due to previous knowledge of its correct functionality (Amigo et al., 2010), the correct number of components for each MCR model was determined by calculating the explained variance of the model and by visual appearance of the chromatographic and spectral profiles as well as the residuals. The explained variance was calculated as follows:

$$\%\mathrm{VAR} = 100 \times \left(1 - \frac{SSE}{SSX}\right),$$

where SSE and SSX account for the sum of the squares of the residuals and the elements in the three-way array, respectively.

(3) Final results: The results obtained were one matrix containing the areas for each one of the compounds modelled in each sample (integrated peaks matrix), another matrix containing the pure mass spectral profiles for each analyte and a set of matrices containing the pure chromatographic profiles for each sample.

(4) Normalization of chromatographic areas and identification of compounds: In order to determine differences in the volatile composition between vinegars, areas of chromatographic peaks were integrated and used as fingerprint signals of samples. After resolving the GC–MS data into pure chromatograms and mass spectra profiles, the integrated peak areas were pre-processed by normalizing all peaks to the peak area associated with the IS (relative areas) to eliminate minor injection discrepancies between samples and extraction efficiency. Moreover, the 'NIST MS Search 2.0' software was used to identify the volatile compounds giving rise to the obtained mass spectra profiles by MCR models. The tentatively identification was based on the mass spectrum matching in the NIST library. Within a total of 192, 228 and 68 components extracted by HSSE, DHS and HS-SPME methods respectively, only those with a probability of matching upper 80% obtained by NIST comparing were selected for this study and confirmed by available retention index of standards (RIs). When standards were not available, the compounds were identified by computer matching to the reference mass spectra from the NIST library and by the comparison of their RIs with the RIs obtained with standards that have been reported in the literature. The remaining compounds were tentatively identified by computer matching to the reference mass spectra from the NIST library and/or through comparisons of their RIs with those of online databases (Flavornet; Pherobase) and the literature. RIs were calculated by using the retention times of n-alkanes under identical conditions for each instrument. All these steps were applied for the three GC–MS data matrices obtained by each sampling method separately.

2.4.2. Statistical analysis

All statistical analysis was carried out by using the PLS_Toolbox 7.9.5 working under MATLAB environment. Analysis of variance (ANOVA), followed by a post hoc comparison test (Tukey's test), and principal component analysis (PCA) were performed.

3. Results and discussion

3.1. MCR analysis

The total ion chromatograms (TIC) of a PDO wine vinegar by the different sampling techniques were shown in Fig. 1. Observing this figure, the complex chemical composition of the analyzed vinegars was clearly showed. Moreover, the presence of a large number of compounds, with overlapping and embedded peaks as well as the baseline/background contribution and low S/N ratio increase the complexity of

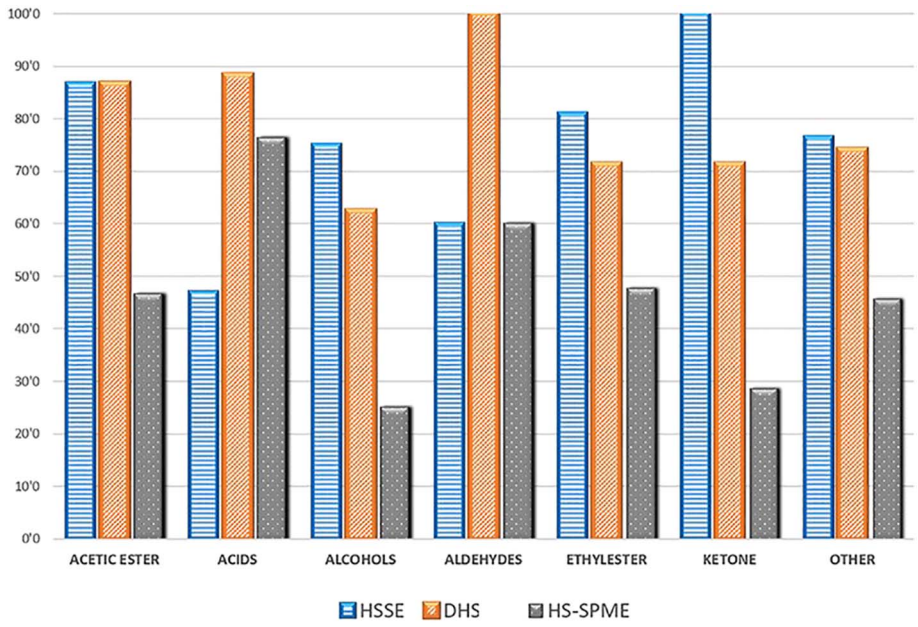

Fig. 3. Percentage of volatile compounds resolved and identified in each sampling method grouped according to their chemical structure.

the studied samples. Due to the overlapped or embedded compounds, direct searching and identification using a MS database became a very difficult and unreliable issue. In fact, by library searching of a single peak, different compounds are obtained at different scan points, meaning that overlapped peaks could not be directly resolved by traditional searches. For all these reasons, the whole TIC of all the samples obtained by each sampling technique separately were divided into 77, 74 and 35 chromatographic regions for HSSE, DHS and HS-SPME respectively, along elution volatile profile. Both segments with single components peaks and segments with overlapping compounds were pretreated using the MCR method described in Section 2.4.1.

In order to show the efficiency and resolution procedure of MCR methodology, two regions of the TIC were highlighted in Fig. 1 (HS-SPME chromatogram) and marked as examples of problematic peak clusters (A, B). The chemical rank (i.e. the number of components in data) of each peak cluster was determined by its morphological score plot that were measurable over the noise level. These two TIC regions and their corresponding MCR resolved elution profiles of each peak cluster were shown in Fig. 2. In this figure, the morphological plot of the first peak cluster example (Fig. 2-A.1) seemed to show only two different components. However, the inspection of the mass spectra along this peak cluster indicated that there could be more than two compounds. In fact, when the MCR was applied, the resolved elution profiles (Fig. 2-A.2) showed the presence of three overlapped compounds, corresponding to diethyl succinate (red peak), isovaleric acid (green peak) and 2-methylbutanoic acid (black peak), respectively. Fig. 2-B.1 showed a peak cluster where two compounds seemed to be overlapped. However, in this case, when MCR was performed (Fig. 2-B.2), this TIC segment illustrated that only presented a single component peak that was finally identified as 2-phenylethyl acetate and it was eluted with a slight shift in retention time so it could be misidentified as two separate peaks.

This is an example that showed how MCR helps in the interpretation of complex chromatograms when phenomena of co-elution, noise and peak alignment take place. Thus, this procedure can be applied to the detection and identification of a higher number of wine vinegar volatile compounds. Moreover, when an automatic integration and identification was performed with this set of samples by using a previous established method by MSD ChemStation, the number of detected and tentatively identified compounds increased from 68 compounds to 81 compounds by using MCR method, as well as the time to process the data is shortened by the use of this chemometric tool.

3.2. Comparison and evaluation of the three sampling methods for the analysis of PDO wine vinegars

After resolving all of peak clusters of each TIC by the same procedure described above, 81 volatile compounds were finally identified including common compounds for the three techniques as well as exclusive compounds extracted by each technique. These compounds were structured in seven groups according to their chemical family (Table 1). The number of resolved and identified volatile compounds differed according to each sampling method: 62 by HSSE-GC–MS, 61 by DHS-GC–MS and 38 by HS-SPME-GC–MS. Fig. 3 showed the percentage of volatile compounds extracted by each technique. In general, it could be observed that for most of the chemical families, a greater number of compounds were extracted by HSSE, followed closely by DHS, except for acids and aldehydes families for which DHS extracted more amount (88.2% and 100%, respectively) than the other two methods. Finally, HS-SPME was the technique that extracted the least amount of volatile compounds in all the chemical families, despite what was expected due to the greater range of polarities of the fiber. These results agreed with previous studies in which a high extraction capability of PDMS stir bars (HSSE) was demonstrated compared with HS-SPME (Bicchi et al., 2002; Loughrin, 2006).

In order to study the significant differences between the chromatographic profiles of the analyzed wine vinegars and between the sapling methods studied, analysis of variance (ANOVA) was performed considering four factors: sampling methods (HSSE, DHS, HS-SPME); the three PDOs; the two categories (Reserva and Pedro Ximenez) and vinegar type (CRE, JRE, MRE, JPX, and MPX). The results obtained (Table 2) showed that the sampling method was the factor that affected the highest number of volatile compounds (67 compounds with $p < 0.05$), which meant that the sampling technique employed affected more the differentiation between samples than their PDO or category, demonstrating the important influence of the sampling in the chromatographic results. This factor was followed by the PDO and the vinegar type's factors, which showed significant differences ($p < 0.05$) only for 32 and 30 compounds, respectively.

As Table 1 showed, there were 27 extracted compounds in common between the three techniques: six acetic esters, eight acids, two alcohols, two aldehydes, seven ethyl esters and two compounds within the group of others. Only taking into account these volatile compounds in common, when ANOVA was performed, all of them showed significant differences between sampling methods ($p < 0.05$). However, this fact

Table 2
Analysis of variance results for the four factors studied: Sampling method, PDO, category and vinegar type.

Compounds		Sampling method[a]		PDO		Category		Vinegar type	
		P-value		P-value		*P*-value		*P*-value	
1	Ethyl acetate	0.00	*	0.07		0.60		0.15	
2	n-propyl acetate	0.94		0.00	*	0.10		0.00	*
3	sec-butyl acetate	HSSE	*	0.05		0.03	*	0.09	
4	Isobutyl acetate	0.00	*	0.00	*	0.66		0.03	*
5	2-methylbutyl acetate	0.24		0.00	*	0.84		0.01	*
6	Isoamyl acetate	0.01	*	0.00	*	0.30		0.02	*
7	Hexyl acetate	0.26		0.00	*	0.10		0.02	*
8	Butyl acetate	DHS	*	0.02	*	0.15		0.09	
9	3-ethoxypropyl acetate	0.00	*	0.33		0.27		0.62	
10	2,3-butanediol diacetate	0.00	*	0.98		0.07		0.35	
11	1,3-propanediol, diacetate	0.00	*	0.08		0.14		0.22	
12	2,3-dihydroxypropyl acetate	0.88		0.10		0.03	*	0.01	*
13	Benzyl acetate	0.00	*	0.99		0.39		0.66	
14	2-phenylethyl acetate	0.00	*	0.50		0.89		0.81	
15	Methyl acetate	HSSE	*	0.07		0.43		0.01	*
16	Acetic acid	0.0043	*	0.78		0.68		0.73	
17	Propanoic acid	0.00	*	1.00		0.24		0.72	
18	Isobutyric acid	0.00	*	0.36		0.54		0.56	
19	Butanoic acid	0.00	*	0.85		0.07		0.47	
20	Isovaleric acid	0.00	*	0.54		0.66		0.85	
21	2-Methylbutanoic acid	HS-SPME	*	0.09		0.00	*	0.00	*
22	Pentanoic acid	0.68		0.10		0.28		0.28	
23	Hexanoic acid	0.00	*	0.40		0.79		0.75	
24	2-Ethylhexanoic acid	0.00	*	0.04	*	0.91		0.16	
25	Octanoic acid	0.00	*	0.80		0.05		0.44	
26	Sorbic acid	0.05	*	0.35		0.46		0.72	
27	Nonanoic acid	0.00	*	0.44		0.00	*	0.03	*
28	Decanoic acid	0.00	*	0.91		0.49		0.87	
29	Dodecanoic acid	0.01	*	0.27		0.02	*	0.04	*
30	Tetradecanoic acid	DHS	*	0.51		0.12		0.49	
31	Pentadecanoic acid	DHS	*	0.36		0.13		0.32	
32	Hexadecanoic acid	DHS	*	0.16		0.07		0.08	
33	Ethanol	HSSE	*	0.01	*	0.98		0.03	*
34	Isoamyl alcohol	0.07		0.00	*	0.17		0.02	*
35	2-Methy-1-butanol	0.03	*	0.01	*	0.78		0.03	*
36	2-ethyl-1-hexanol	HSSE	*	0.43		0.85		0.08	
37	2-phenylethanol	0.00	*	0.49		0.90		0.80	
38	1-hexanol	DHS	*	0.02	*	0.16		0.09	
39	1,3-propanediol	DHS	*	0.17		0.11		0.11	
40	2-furanmethanol	HSSE	*	0.29		0.12		0.30	
41	Benzaldehyde	0.00	*	0.10		0.07		0.21	
42	Furfural	0.00	*	0.36		0.26		0.63	
43	5-Methylfurfural	0.00	*	0.85		0.82		0.66	
44	5-acetoxymethyl-2-furaldehyde	DHS	*	0.20		0.00	*	0.00	*
45	5-hydroxymethylfurfural	0.00	*	0.23		0.00	*	0.00	*
46	Ethyl propanoate	0.87		0.00	*	0.05		0.00	*
47	Ethyl isobutyrate	0.02	*	0.00	*	0.15		0.00	*
48	Ethyl butyrate	0.02	*	0.00	*	0.04	*	0.01	*
49	Ethyl 2-methylbutyrate	0.00	*	0.00	*	0.46		0.00	*
50	Ethyl isovalerate	0.01	*	0.00	*	0.72		0.00	*
51	Ethyl pentanoate	DHS	*	0.01	*	0.17		0.08	
52	Ethyl hexanoate	0.10		0.01	*	0.27		0.04	*
53	Ethyl 3-ethoxypropanoate	DHS	*	0.02	*	0.17		0.12	
54	Ethyl heptanoate	HSSE	*	0.02	*	0.53		0.13	
55	Ethyl octanoate	0.13		0.01	*	0.22		0.05	
56	Ethyl sorbate	0.20		0.01	*	0.22		0.05	
57	Ethyl decanoate	0.06		0.01	*	0.94		0.01	*
58	Ethyl benzoate	0.03	*	0.91		0.01	*	0.06	
59	Diethyl succinate	0.08		0.01	*	0.22		0.07	
60	Ethyl phenylacetate	0.01	*	0.02	*	0.53		0.08	
61	Ethyl dodecanoate	0.66		0.07		0.76		0.08	
62	Diethyl malate	DHS	*	0.01	*	0.28		0.08	
63	Methyl hexadecanoate	HSSE	*	0.85		0.38		0.66	
64	Ethyl hexadecanoate	HSSE	*	0.54		0.19		0.12	
65	Ethyl furoate	0.00	*	0.07		0.46		0.25	
66	Ethyl hydrogen succinate	HS-SPME	*	0.07		0.66		0.26	
67	2,3-butanedione	HSSE	*	0.35		0.02	*	0.01	*
68	Methyl isobutyl ketone	HSSE	*	0.14		0.07		0.03	*
69	Acetoin	0.00	*	0.65		0.72		0.80	
70	1-hydroxy-2-propanone	HSSE	*	0.92		0.99		0.99	
71	3-nonanone	HSSE	*	0.02	*	0.12		0.09	
72	2-acetoxy-3-butanone	HSSE	*	0.10		0.28		0.01	*
73	2-acethylfuran	0.00	*	0.56		0.71		0.79	
74	2(5H)-furanone	HSSE	*	0.52		0.31		0.68	
75	Linalool 3,7-oxide	HSSE	*	0.97		0.38		0.60	
76	TDN	0.32		0.12		0.02	*	0.01	*
77	2,4,5-Trimethyl-1,3-Dioxolane	0.37		0.00	*	0.01	*	0.00	*

(continued on next page)

Table 2 *(continued)*

Compounds		Sampling method[a]		PDO		Category		Vinegar type	
		P-value		P-value		*P*-value		*P*-value	
78	1,4:3,6-dianhydro-α-d-glucopyranose	0.00	*	0.13		0.38		0.22	
79	Methyl salicylate	0.00	*	0.00	*	0.51		0.00	*
80	Isopropyl myristate	HSSE	*	0.02	*	0.04	*	0.04	*
81	4-ethylguaiacol	0.00	*	0.03	*	0.40		0.10	

* Values are significant at $p < 0.05$.

[a] A name of a sampling method in this column means that this compound was only extracted by that method.

did not occur with the other three factors (PDO, Category and Vinegar type) reaffirming that the effect of the sampling method used is greater than the produced by the different characteristics of samples.

According to the results obtained in the ANOVA, in which the highest significant difference was obtained by the sampling method factor, a post-hoc comparison Tukey's test was applied to this factor, in order to compare each sampling method with the others. The statistical results (mean relative areas of the compounds of all the samples and standard deviations) were presented in Table 3. Several significant differences were found in the majority of the compounds according to the three sampling methods. As can be observed, excepting 4 compounds (2-methylbutyl acetate (5); ethyl hexanoate (52); diethyl succinate (59), and 1,1,6-Trimethyl-1,2-dihydronaphthalene (TDN)(76)), the rest of compounds showed significant differences between methods, highlighting that the most of them seemed to present higher mean relative areas by DHS extraction than by the other two sampling methods (Table 3). In fact, when the total average of the areas obtained were calculated, the mean relative areas of the total of extracted compounds obtained by DHS were almost twice of those obtained by HSSE and the triple for HS-SPME. This effect could be explained by the fact that in DHS, the inert gas used to sweep the headspace of the sample favors the extraction of volatile compounds. However, in HS methods, the aliquot analyzed is taken directly from the vapor phase in equilibrium with the sample by heating the vial. More advantages and disadvantages about these methods according to several author's experiences were summarized by Morales et al. (2013).

In addition to these differences between techniques, it should be also noted that the resulting volatile profile of each sample (regarded as the percentage of the total relative areas for each chemical family) changed depending on the sampling method used (Fig. 4). That could be explained by the physical, chemical and methodological differences between the three sampling methods, within which the amount and type of extraction polymer, the extractability and their sensitivity would had an important role (Morales et al., 2013). Taking into account that one of the fundamental differences between HSSE and HS-SPME is the amount of adsorbent they contained, around 100–1000 times greater in the coated magnetic stir bars of PDMS than in HS-SPME fibers (Baltussen et al., 1999), the results obtained were as expected. In our case, the amount of PDMS in SPME fiber was in the order of 0.5 μL whereas in HSSE the amount of PDMS was 55 μL. In regards to DHS, the obtained chromatographic profiles were similar to those of HSSE in qualitative terms, since the two adsorbents used (Tenax and PDMS, respectively) had a similar polarity, having both an apolar character. Thus, for example, compounds that usually are presented in high relative areas in wine vinegar samples, such as ethyl acetate and isoamyl alcohol, were only extracted by these two methods (Callejón et al., 2008; Callejón et al., 2008; Tesfaye et al., 2010). However, the relative areas obtained for many of the extracted compounds were slightly higher by DHS than by HSSE (Table 1) in accordance with the above-mentioned differences in their methodologies (i.e. amount of polymer, headspace extraction methodology, etc.) Regarding HS-SPME, two compounds were able to be identified only in this method and not by the other methods, one acid (2-methylbutanoic acid (21)) and one ethyl ester (ethyl hydrogen succinate (66)).

3.3. Evaluation of the three sampling methods in their ability of wine vinegar differentiation

One of the aims of this study was to assess how each sampling method influences on the characterization and differentiation of different types of wine vinegars, belonging to different PDOs and categories. In this context, and acknowledging that there might not be a significant amount of samples, the dataset obtained by each sampling method was submitted to principal component analysis (PCA) in order to easily explore and visualize the chromatographic results and ascertain the degree of differentiation between samples analyzed by the three sampling methods. Fig. 5 shows the scores plots obtained by the principal components (PCs) with the most significant influence upon the PCA that showed better separation of samples. From the three PCA models, 6 significant PCs were chosen on the basis of Kaiser's criterion (eigenvalues higher than 1.0 are chosen) accounting for 86.88%, 88.84% and 95.29% of total variability from HSSE, DHS and HS-SPME PCA models, respectively. Firstly, regarding the scores plots in Fig. 5, which included the samples in duplicate, good clustering for each wine vinegar sample duplicates indicated that the sampling method, the GC–MS analysis and the MCR procedures were robust enough. Secondly, considering the three different PDOs, as could be seen in Fig. 5-A, the worst differentiation between PDOs seemed to perfectly be obtained by DHS model due to the presence of overlapping between some samples in the negative side of the first principal component (PC1). In spite of this, it could be observed that “Vinagre de Jerez” PDO was well grouped and separated from the rest in the three PCA modes, regardless of the sampling method employed. Moreover, the scores plot obtained by HSSE and HS-SPME shows that “Vinagre de Montilla-Moriles” samples were also differentiated from the rest, placed in the positive side of PC4 and PC3, respectively. On the contrary, none of the sampling methods was able to separate “Vinagre de Condado de Huelva” PDO, showing a higher degree of overlapping with other PDOs. On the other hand, in regards to the separation between categories (Reserva and Pedro Ximenez) showed in Fig. 5-B, HSSE and HS-SPME models showed a good differentiation between the categories, being the last one, the method which showed the best differentiation. Once again, the worst discrimination between these two categories was obtained by DHS.

The PCA loadings of the principal components that well separated the samples were inspected (Table 4) in order to investigate the basis for the observed differences between the PDOs. It was observed that loadings on PC1 were mainly positive in the three PCA models, in which higher weight was observed for the family of acetic esters, ethyl esters and acids. These volatile compounds showed more relevance in the differentiation between categories than between PDOs (Fig. 5-B). With respect to PC4 and PC3 loadings, variables with a higher weight were some acids (e.g., 2-ethylhexanoic acid (24) with negative values of PC4), ethyl esters (e.g. ethyl decanoate (57) with positive values of PC4) and benzaldehyde (41). These compounds showed positive values of PC3 and PC4 (Table 4), which made them relevant in the

Table 3
Mean relative areas and standard deviation of all the volatile compounds. Results from Tukey's test according to the variable "Sampling methods".

	Sampling method	*HSSE*		*DHS*		*HS-SPME*	
N	Compound	Mean	± SD	Mean	± SD	Mean	± SD
1	Ethyl acetate	44.17 c	13.91	13.37 b	11.14	nd a	–
2	n-Propyl acetate	0.68 b	0.76	0.69 b	0.84	nd a	–
3	sec-Butyl acetate	0.12 b	0.15	nd a	–	nd a	–
4	Isobutyl acetate	3.80 b	2.89	2.09 a	1.59	0.94 a	0.99
5	2-Methylbutyl acetate	5.01 a	3.36	5.39 a	3.96	3.63 a	2.82
6	Isoamyl acetate	20.24 ab	18.41	32.12 b	35.77	8.05 a	9.26
7	Hexyl acetate	0.20 b	0.32	0.49 b	1.11	nd a	–
8	Butyl acetate	nd a	–	0.69 b	0.94	nd a	–
9	3-Ethoxypropyl acetate	0.07 b	0.03	1.04 c	0.92	nd a	–
10	2,3-Butanediol diacetate	0.17 b	0.09	1.02 c	0.75	nd a	–
11	1,3-Propanediol, diacetate	0.11 a	0.10	1.66 b	1.82	0.25 a	0.12
12	2,3-Dihydroxypropyl acetate	nd a	–	0.25 b	0.31	0.27 b	0.32
13	Benzyl acetate	0.15 a	0.09	0.95 b	0.72	0.37 a	0.52
14	2-Phenylethyl acetate	4.80 a	2.09	13.17 b	11.28	9.81 ab	6.97
15	Methyl acetate	0.94 b	0.29	nd a	–	nd a	–
16	Acetic acid	11.34 a	4.19	33.64 b	14.18	6.85 a	3.87
17	Propanoic acid	nd a	–	2.11 c	1.28	0.48 b	0.25
18	Isobutyric acid	0.31 a	0.14	3.83 b	1.85	0.74 a	0.36
19	Butanoic acid	0.12 a	0.11	8.27 b	8.11	0.85 a	0.48
20	Isovaleric acid	3.48 a	1.10	20.81 c	8.51	9.13 b	6.09
21	2-Methylbutanoic acid	nd a	–	nd a	–	0.81 b	0.52
22	Pentanoic acid	nd a	–	0.21 b	0.15	0.20 b	0.14
23	Hexanoic acid	0.10 a	0.04	2.65 c	1.56	1.37 b	0.94
24	2-Ethylhexanoic acid	0.04 a	0.03	0.15 b	0.14	0.13 b	0.09
25	Octanoic acid	0.11 a	0.06	2.53 b	1.55	3.74 c	2.25
26	Sorbic acid	nd a	–	0.06 b	0.08	0.11 c	0.07
27	Nonanoic acid	nd a	–	0.20 b	0.14	1.13 c	0.86
28	Decanoic acid	0.13 a	0.08	6.78 b	5.09	7.49 b	4.89
29	Dodecanoic acid	nd a	–	0.15 b	0.15	0.92 c	1.26
30	Tetradecanoic acid	nd a	–	0.18 c	0.16	nd a	–
31	Pentadecanoic acid	nd a	–	0.08 b	0.07	nd a	–
32	Hexadecanoic acid	nd a	–	0.28 b	0.21	nd a	–
33	Ethanol	4.35 b	2.62	nd a	–	nd a	–
34	Isoamyl alcohol	4.07 b	3.70	8.87 b	10.81	nd a	–
35	2-Methy-1-butanol	2.93 ab	1.56	4.15 b	2.94	2.32 a	1.63
36	2-Ethylhexanol	0.12 b	0.05	nd a	–	nd a	–
37	2-Phenylethanol	1.42 a	0.55	18.53 b	15.62	7.59 a	6.53
38	1-Hexanol	nd a	–	1.66 b	3.36	nd a	–
39	1,3-Propanediol	nd a	–	0.07 b	0.06	nd a	–
40	2-Furanmethanol	0.34 b	0.16	nd a	–	nd a	–
41	Benzaldehyde	0.58 a	0.67	8.03 b	12.86	0.60 a	0.35
42	Furfural	1.13 b	0.63	21.59 c	18.09	nd a	–
43	5-Methylfurfural	0.08 a	0.05	0.77 b	0.60	0.13 a	0.16
44	5-Acetoxymethyl-2-furaldehyde	nd a	–	0.04 b	0.04	nd a	–
45	5-Hydroxymethylfurfural	nd a	–	0.11 b	0.10	0.44 c	0.44
46	Ethyl propanoate	0.65 b	0.72	0.61 b	0.90	nd a	–
47	Ethyl isobutyrate	0.87 c	0.90	0.35 b	0.36	nd a	–
48	Ethyl butyrate	0.62 ab	0.78	1.50 b	2.49	0.13 a	0.10
49	Ethyl 2-methylbutyrate	0.52 b	0.54	0.23 a	0.24	0.13 a	0.13
50	Ethyl isovalerate	4.68 b	4.08	3.33 a b	2.72	1.60 a	1.44
51	Ethyl pentanoate	nd a	–	0.21 b	0.27	nd a	–
52	Ethyl hexanoate	0.60 a	0.86	2.36 a	5.53	0.31 a	0.33
53	Ethyl 3-ethoxypropanoate	nd a	–	0.28 b	0.46	nd a	–
54	Ethyl heptanoate	0.02 b	0.02	nd a	–	nd a	–
55	Ethyl octanoate	0.51 b	0.85	3.36 b	8.22	nd a	–
56	Ethyl sorbate	0.07 b	0.10	0.48 b	1.39	nd a	–
57	Ethyl decanoate	0.08 b	0.13	nd a	–	0.15 b	0.11
58	Ethyl benzoate	0.07 b	0.05	nd a	–	0.14 c	0.12
59	Diethyl succinate	0.27 a	0.45	5.70 a	14.69	0.38 a	0.41
60	Ethyl phenylacetate	0.73 a	0.62	4.52 b	6.14	1.62 a	1.65
61	Ethyl dodecanoate	0.01 b	0.01	0.02 b	0.03	nd a	–
62	Diethyl malate	nd a	–	0.02 b	0.04	nd a	–
63	Methyl hexadecanoate	0.06 b	0.05	nd a	–	nd a	–
64	Ethyl hexadecanoate	0.02 b	0.02	nd a	–	nd a	–
65	Ethyl furoate	0.06 a	0.04	0.63 b	0.73	0.09 a	0.08
66	Ethyl hydrogen succinate	nd a	–	nd a	–	0.11 b	0.10
67	2,3-Butanedione	1.44 b	0.85	nd a	–	nd a	–
68	Methyl Isobutyl ketone	0.14 b	0.19	nd a	–	nd a	–
69	Acetoin	1.03 b	0.81	5.21 c	3.06	nd a	–
70	1-Hydroxy-2-propanone	1.55 b	0.61	nd a	–	nd a	–
71	3-Nonanone	0.02 b	0.03	nd a	–	nd a	–
72	2-Acetoxy-3-butanone	0.27 b	0.20	nd a	–	nd a	–
73	2-Acethylfuran	0.11 b	0.03	1.44 c	0.75	nd a	–
74	2(5H)-Furanone	0.21 b	0.09	nd a	–	nd a	–
75	Linalool 3,7-oxide	0.08 b	0.06	nd a	–	nd a	–
76	TDN	0.25 a	0.40	0.22 a	0.25	0.59 a	1.41
77	2,4,5-Trimethyl-1,3-Dioxolane	1.13 b	1.24	0.80 b	1.09	nd a	–

(continued on next page)

Table 3 *(continued)*

	Sampling method	*HSSE*		*DHS*		*HS-SPME*	
N	Compound	Mean	± SD	Mean	± SD	Mean	± SD
78	1,4:3,6-Dianhydro-α-D-glucopyranose	0.06 c	0.03	0.02 b	0.03	nd a	–
79	Methyl salicylate	0.03 a	0.03	0.15 b	0.17	0.07 ab	0.06
80	Isopropyl myristate	0.05 b	0.05	nd a	–	nd a	–
81	4-Ethylguaiacol	0.04 b	0.02	0.23 c	0.15	nd a	–

Different letters in different columns indicate significant differences according to Tukey's test ($P < 0.05$). From letter 'a' to letter 'c' indicates increasing concentrations.
nd: peak not detected

differentiation between "Vinagre de Jerez" and "Montilla-Moriles" PDOs by HS-SPME and HSSE, respectively. Moreover, in DHS model, other compounds that mainly accounted for this variance were 5-acetoxymethyl-2-furaldehyde (44) and 5-hydroxymethylfurfural (45). "Vinagre de Condado de Huelva" PDO seemed to be more characterized by some compounds such as benzaldehyde (41), some acids as

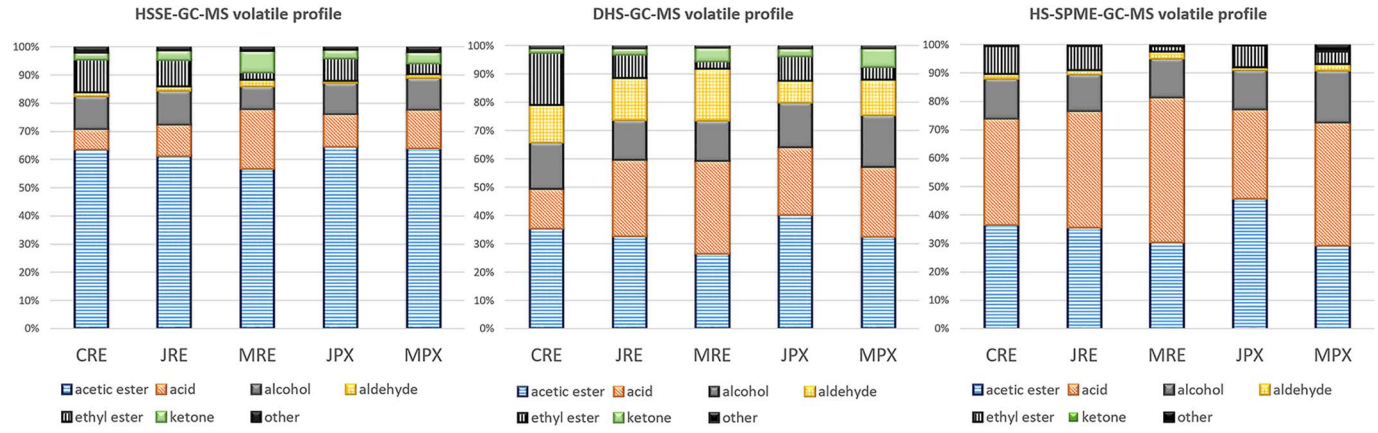

Fig. 4. Volatile profile of the PDO wine vinegars samples obtained by HSSE-GC-MS, DHS GC-MS and HS-SPME-GC-MS analysis. Each graphic shows the percentage of the total relative area obtained for each chemical family by each extraction method.

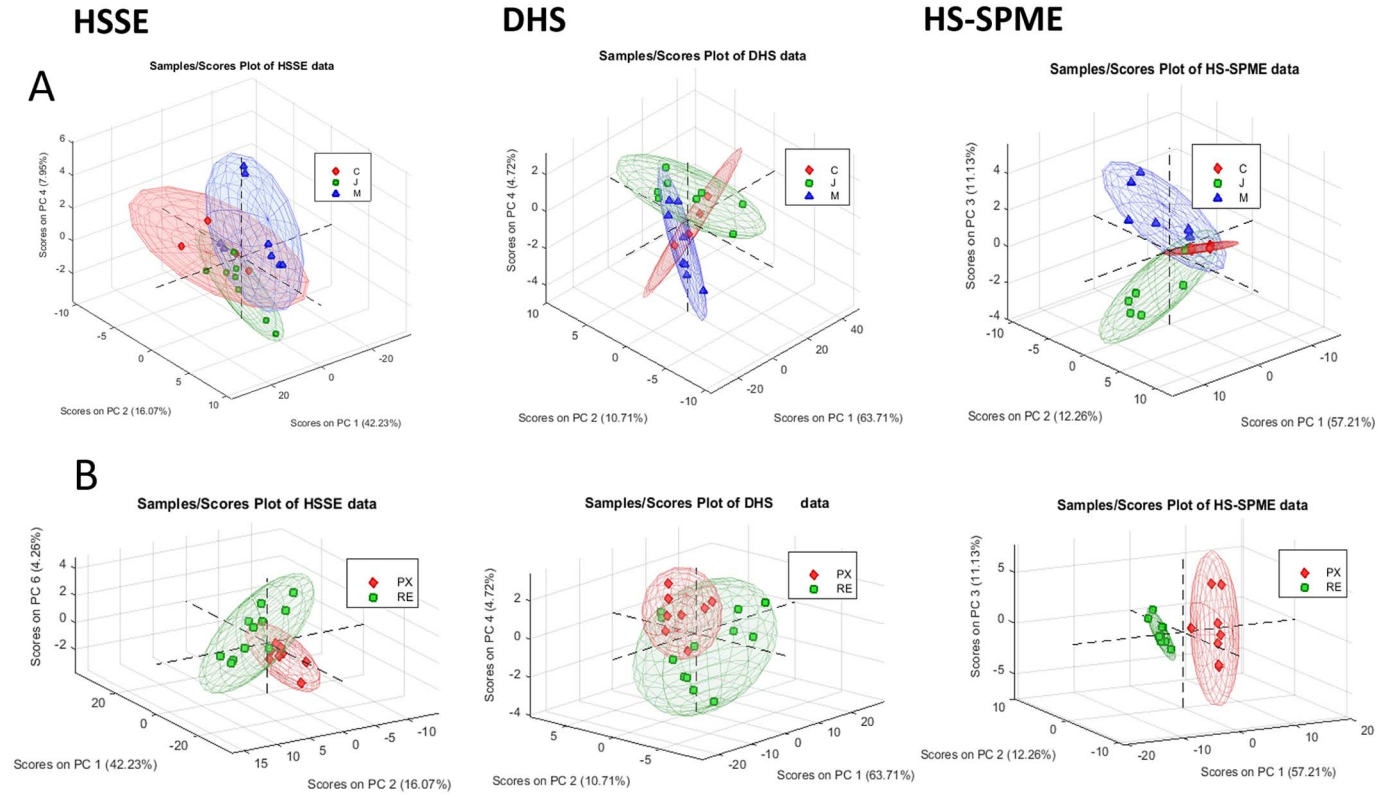

Fig. 5. Scores plot of the principal components obtained by a PCA of the dataset obtained by each extraction method. Samples according the PDO (A) and the two categories studied (B) are shown.

Table 4
Loadings plot of the principal components obtained by PCA of each sampling method, according to the volatile compounds extracted and their chemical families.

N	Family	Compounds	HSSE				DHS			HS-SPME		
			PC 1 42.2%	PC 2 16.1%	PC 4 7.9%	PC 6 4.3%	PC 1 63.7%	PC 2 10.7%	PC 4 4.8%	PC 1 57.2%	PC 2 12.2%	PC 3 11.1%
1	Acetic ester	Ethyl Acetate	0.09	−0.10	−0.08	−0.28	0.15	−0.04	0.15	–	–	–
2	Acetic ester	n-Propyl acetate	0.18	0.00	−0.01	−0.06	0.15	−0.07	0.10	–	–	–
3	Acetic ester	sec-Butyl acetate	0.17	0.04	0.11	0.01	–	–	–	–	–	–
4	Acetic ester	Isobutyl acetate	0.18	−0.01	−0.04	−0.05	0.14	−0.08	0.14	0.18	0.13	−0.10
5	Acetic ester	2-Methylbutyl acetate	0.19	−0.05	−0.03	0.04	0.16	0.00	0.04	0.20	0.05	−0.06
6	Acetic ester	Isoamyl acetate	0.19	−0.04	0.02	0.00	0.16	0.00	0.02	0.17	0.20	−0.04
7	Acetic ester	Hexyl acetate	0.18	−0.08	0.04	0.10	0.15	0.09	−0.06	–	–	–
8	Acetic ester	Butyl acetate	–	–	–	–	0.16	0.01	−0.03	–	–	–
9	Acetic ester	3-Ethoxypropyl acetate	0.10	0.22	0.08	0.04	0.15	−0.12	−0.02	–	–	–
10	Acetic ester	2,3-Butanediol diacetate	0.04	0.25	0.13	0.02	0.10	−0.19	−0.24	–	–	–
11	Acetic ester	1,3-Propanediol, diacetate	0.06	0.20	0.10	−0.04	0.15	−0.02	−0.13	0.18	0.09	0.07
12	Acetic ester	2,3-Dihydroxypropyl acetate	–	–	–	–	0.09	−0.03	0.05	−0.10	0.06	0.04
13	Acetic ester	Benzyl acetate	0.05	0.12	0.20	−0.04	0.13	−0.12	0.04	0.14	−0.05	0.26
14	Acetic ester	2-Phenylethyl acetate	0.13	0.21	−0.02	0.05	0.15	−0.09	−0.07	0.21	0.02	0.05
15	Acetic ester	Methyl acetate	0.07	0.06	−0.24	−0.06	–	–	–	–	–	–
16	Acid	Acetic acid	0.01	0.21	−0.10	−0.16	0.04	0.29	0.12	0.19	−0.10	−0.01
17	Acid	Propanoic acid	–	–	–	–	0.09	−0.11	−0.25	0.20	0.00	0.05
18	Acid	Isobutyric acid	0.04	0.24	−0.22	−0.06	0.05	−0.23	0.03	0.20	−0.04	−0.17
19	Acid	Butanoic acid	0.04	0.26	−0.05	−0.17	0.10	−0.21	−0.16	0.20	−0.07	−0.10
20	Acid	Isovaleric acid	−0.03	0.28	−0.12	0.06	0.14	−0.10	0.05	0.19	−0.19	0.00
21	Acid	2-Methylbutanoic acid	–	–	–	–	–	–	–	0.17	−0.15	−0.18
22	Acid	Pentanoic acid	–	–	–	–	0.10	−0.19	−0.10	0.21	−0.07	−0.09
23	Acid	Hexanoic acid	0.02	0.26	−0.04	−0.19	0.14	−0.07	0.00	0.21	−0.07	−0.03
24	Acid	2-Ethylhexanoic acid	0.04	0.09	−0.22	0.05	0.13	−0.20	0.06	0.19	0.09	−0.10
25	Acid	Octanoic acid	0.10	0.19	−0.12	−0.11	0.13	0.13	−0.04	0.19	−0.17	0.10
26	Acid	Sorbic Acid	–	–	–	–	0.01	0.25	0.09	0.02	0.37	0.20
27	Acid	Nonanoic acid	–	–	–	–	0.14	0.15	−0.05	0.19	−0.17	0.11
28	Acid	Decanoic acid	0.10	0.16	−0.02	−0.14	0.13	0.20	−0.09	0.13	−0.05	0.26
29	Acid	Dodecanoic acid	–	–	–	–	0.07	0.28	−0.02	0.13	−0.09	0.35
30	Acid	Tetradecanoic acid	–	–	–	–	0.08	0.24	−0.07	–	–	–
31	Acid	Pentadecanoic acid	–	–	–	–	0.07	0.24	−0.02	–	–	–
32	Acid	Hexadecanoic acid	–	–	–	–	0.09	0.18	0.02	–	–	–
33	Alcohol	Ethanol	0.16	−0.03	−0.08	−0.16	–	–	–	–	–	–
34	Alcohol	Isoamyl alcohol	0.18	−0.01	0.09	0.01	0.15	0.01	−0.01	–	–	–
35	Alcohol	2-Methy-1-butanol	0.18	0.00	0.07	0.04	0.16	0.00	0.00	0.20	0.09	0.15
36	Alcohol	2-Ethyl-1-hexanol	0.10	−0.03	0.10	0.00	–	–	–	–	–	–
37	Alcohol	2-Phenylethanol	0.12	0.19	0.11	0.11	0.16	−0.04	−0.04	0.19	−0.01	0.22
38	Alcohol	1-Hexanol	–	–	–	–	0.15	0.08	−0.06	–	–	–
39	Alcohol	1,3-Propanediol	–	–	–	–	0.10	−0.24	0.11	–	–	–
40	Alcohol	2-Furanmethanol	0.13	−0.03	−0.09	0.08	–	–	–	–	–	–
41	Aldehyde	Benzaldehyde	0.12	0.09	0.12	0.04	0.15	0.04	−0.21	0.07	0.04	0.26
42	Aldehyde	Furfural	0.08	−0.11	−0.17	0.10	0.13	0.15	0.01	–	–	–
43	Aldehyde	5-Methylfurfural	0.05	−0.17	−0.05	0.04	0.12	0.15	−0.02	0.10	−0.20	0.15
44	Aldehyde	5-Acetoxymethyl-2-furaldehyde	–	–	–	–	−0.02	0.18	0.42	–	–	–
45	Aldehyde	5-Hydroxymethylfurfural	–	–	–	–	−0.01	0.07	0.46	0.17	−0.18	0.13
46	Ethyl ester	Ethyl propanoate	0.19	−0.02	0.03	−0.03	0.15	0.02	0.03	–	–	–
47	Ethyl ester	Ethyl isobutyrate	0.19	−0.04	−0.06	−0.05	0.15	−0.01	0.11	–	–	–
48	Ethyl ester	Ethyl butyrate	0.19	0.04	−0.02	−0.06	0.15	−0.02	0.00	0.10	0.28	−0.20
49	Ethyl ester	Ethyl 2-methylbutyrate	0.18	−0.06	−0.06	−0.03	0.15	0.00	0.12	0.17	0.03	−0.30
50	Ethyl ester	Ethyl isovalerate	0.18	−0.09	−0.09	−0.02	0.15	0.01	0.19	0.19	0.02	−0.23
51	Ethyl ester	Ethyl pentanoate	–	–	–	–	0.16	0.00	0.02	–	–	–
52	Ethyl ester	Ethyl hexanoate	0.18	−0.07	0.02	0.00	0.15	0.07	−0.04	0.17	0.21	−0.16
53	Ethyl ester	Ethyl 3-ethoxypropanoate	–	–	–	–	0.16	0.02	−0.05	–	–	–
54	Ethyl ester	Ethyl heptanoate	0.18	−0.06	0.02	−0.02	–	–	–	–	–	–
55	Ethyl ester	Ethyl octanoate	0.18	−0.08	0.04	0.03	0.15	0.08	−0.05	–	–	–
56	Ethyl ester	Ethyl sorbate	0.17	−0.07	0.11	0.05	0.15	0.09	−0.08	–	–	–
57	Ethyl ester	Ethyl decanoate	0.17	−0.10	0.07	0.06	–	–	–	0.10	0.23	0.15
58	Ethyl ester	Ethyl benzoate	0.19	−0.01	0.10	−0.04	–	–	–	0.20	0.06	0.16
59	Ethyl ester	Diethyl succinate	0.18	−0.01	0.05	0.01	0.15	0.06	−0.04	0.10	0.35	0.10
60	Ethyl ester	Ethyl phenylacetate	0.15	0.03	−0.16	−0.06	0.16	−0.02	0.06	0.15	−0.08	−0.24
61	Ethyl ester	Ethyl dodecanoate	−0.02	−0.03	0.36	−0.16	0.14	0.06	−0.01	–	–	–
62	Ethyl ester	Diethyl malate	–	–	–	–	0.14	0.05	0.01	–	–	–
63	Ethyl ester	Methyl hexadecanoate	0.00	0.05	−0.03	−0.06	–	–	–	–	–	–
64	Ethyl ester	Ethyl hexadecanoate	0.02	−0.02	0.33	−0.06	–	–	–	–	–	–
65	Ethyl ester	Ethyl furoate	0.17	0.03	0.01	−0.01	0.16	−0.01	0.04	0.21	0.03	0.03
66	Ethyl ester	Ethyl hydrogen succinate	–	–	–	–	–	–	–	0.05	0.42	0.06
67	Ketone	2,3-Butanedione	0.09	0.18	0.17	0.12	–	–	–	–	–	–
68	Ketone	Methyl Isobutyl ketone	0.08	−0.13	−0.11	0.34	–	–	–	–	–	–
69	Ketone	Acetoin	−0.03	0.16	0.07	0.32	0.06	−0.07	−0.10	–	–	–
70	Ketone	1-Hydroxy-2-propanone	0.09	0.03	−0.01	−0.22	–	–	–	–	–	–
71	Ketone	3-Nonanone	0.18	−0.06	0.06	0.08	–	–	–	–	–	–
72	Ketone	2-Acetoxy-3-butanone	−0.04	0.21	0.10	0.28	–	–	–	–	–	–
73	Ketone	2-Acethylfuran	0.06	−0.18	0.02	−0.06	0.13	0.07	0.20	–	–	–
74	Ketone	2(5H)-Furanone	0.12	−0.03	−0.08	0.02	–	–	–	–	–	–
75	Other	Linalool 3,7-oxide	0.01	−0.18	−0.18	−0.16	–	–	–	–	–	–
76	Other	TDN	−0.04	−0.15	−0.07	−0.24	0.03	0.14	0.03	0.04	−0.23	0.08

(continued on next page)

Table 4 *(continued)*

N	Family	Compounds	HSSE				DHS			HS-SPME		
			PC 1 42.2%	PC 2 16.1%	PC 4 7.9%	PC 6 4.3%	PC 1 63.7%	PC 2 10.7%	PC 4 4.8%	PC 1 57.2%	PC 2 12.2%	PC 3 11.1%
77	Other	2,4,5-Trimethyl-1,3-Dioxolane	0.18	0.00	0.09	0.02	0.15	0.03	− 0.08	–	–	–
78	Other	1,4:3,6-Dianhydro-α-D-glucopyranose	0.06	− 0.10	− 0.11	0.28	0.06	− 0.26	0.24	–	–	–
79	Other	Methyl salicylate	0.05	0.13	− 0.26	0.00	0.07	− 0.24	0.17	0.17	− 0.11	− 0.24
80	Other	Isopropyl myristate	− 0.06	0.01	0.17	− 0.32	–	–	–	–	–	–
81	Other	4-Ethylguaiacol	0.05	0.09	− 0.27	0.11	0.12	− 0.08	0.21	–	–	–

propanoic acid (17) and decanoic acid (28) and some ethyl esters as ethyl butyrate (48) according to the loadings plot showed in Table 4. Regarding the separation of both categories, PC1 had an important role in this differentiation, especially in HS-SPME model. Taking into account that this method extracted the least number of compounds it could be deduced that all of the compounds extracted by this technique, with positive PC1 loadings and negative PC2 loadings, would be those that characterize the Pedro Ximenez category (Table 4). Some of those compounds were 2-phenylethyl acetate (14), 2-phenylethanol (37), 5-methylfurfural (43), 5-hydroxymethylfurfural (45), TDN (76) and almost all the extracted acids. Regarding HSSE and DHS, "Pedro Ximenez" samples were characterized by volatile compounds with negative loadings of PC1 and PC2 in both techniques, and negative PC6 loadings and positive PC4 loadings, respectively. In this two sampling methods, ethyl acetate (1), isobutyl acetate (4) or ethyl dodecanoate (61) were some of the compounds involved in this differentiation. It should be highlighted that the "Pedro Ximenez" category differs from the aged category by their different production process. Thus, they are produced by the addition of must of raisined "Pedro Ximenez" grapes (in the case of "Vinagre de Montilla-Moriles") or the addition of "Pedro Ximenez" wine to the vinegar (in "Vinagre de Jerez" PDO) (Ríos-Reina et al., 2017; Ríos-Reina, Callejón, Oliver-Pozo, Amigo, & García-González, 2017). This addition could increase the residual alcohol in the wine vinegar, which had showed a clearly influence in their final content of volatile compounds, mainly ethyl acetate (Tesfaye, Morales, García-Parrilla, & Troncoso, 2002). Furthermore, as it can be observed in Table 1, the content of ethanol (33) as well as the total amount of ethyl esters, was higher in "Pedro Ximenez" wine vinegars (MPX and JPX) than in the Reserva samples of their corresponding PDOs (MRE and JRE).

After applying the multivariate data analysis, the compounds that showed an important relevance in the wine vinegar characterization were generally consistent with previous reported compounds in high quality wine vinegars by different extraction methods prior to GC–MS analysis (Callejón et al., 2008; Callejón et al., 2008; Castro Mejías et al., 2002; Chinnici et al., 2009; Marrufo-Curtido et al., 2012; Pizarro et al., 2008). Nevertheless, this study demonstrated that the wine vinegar volatile profile obtained by GC–MS analysis highly depends on the previous sampling technique used.

4. Conclusions

According to the aims of this study, the physical, chemical and methodological differences between the three sampling methods have demonstrated to have an important influence in the volatile composition obtained for each wine vinegar. Hence, not all the sampling methods applied prior to GC–MS analysis are equally suitable for the differentiation and characterization of high quality wine vinegars and their aged and sweet categories. Greater number of compounds were extracted by HSSE for most of the chemical families, followed closely by DHS. HS-SPME was the technique that extracted the least amount of volatile compounds in all the chemical families. Taking into account that one of the main needs of the wineries is the characterization of their vinegars, the knowledge of the maximum information about the wine vinegar is essential to perform this issue. In terms of differentiation, HSSE-GC–MS and HS-SPME-GC–MS have shown a better discrimination of PDOs and categories, although HS-SPME showed a clearer discrimination between aged and sweet categories.

A suitable selection of the sampling method in combination with chemometric methodologies, such as multiple curve resolution, could be successfully applied as a quality control tool for PDO wine vinegars and some of their most commercialized categories. Furthermore, MCR methodology has demonstrated to reduce the associated problems to the GC–MS analysis of complex mixtures such as high quality food products and to streamlines data processing. Although it should be noted that this is only a feasibility study, once the influence of this prior step has been demonstrated, the encouraging results obtained justify the consideration of a similar approach in future in order to better evaluate its actual performance and to broaden the field of application to a wider range of vinegar types. The applicability of the proposed methodology by any of the studied sampling methods requires further research. Thus, the development of classification models and the inclusion of a broader number of samples of each PDO and category are being carried out.

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Table 1. Mean relative area of the volatile compounds extracted in the different wine vinegar samples by each sampling method.

Nº	Volatile compounds[a]	ID[b]	RI[c]	CRE						JRE						MRE						JPX						MPX					
				HSSE		DHS		HSSPME		HSSE		DHS		HSSPME		HSSE		DHS		HSSPME		HSSE		DHS		HSSPME		HSSE		DHS		HSSPME	
				ARm[d]	±SD	ARm	±SD	ARm	±SD	ARm	±SD	ARm	±SD	ARm	±SD	ARm	±SD	ARm	±SD	ARm	±SD	ARm	±SD	ARm	±SD	ARm	±SD	ARm	±SD	ARm	±SD	ARm	±SD
	ACETIC ESTERS																																
1	Ethyl Acetate	A	903	48.2	8.16	25.4	21.1	nd	-	42.5	0.61	15.7	7.90	nd	-	29.4	21.5	2.95	3.70	nd	-	54.7	17.9	20.7	0.90	nd	-	39.0	0.72	8.68	2.90	nd	-
2	n-Propyl acetate	A	947	1.47	1.31	1.55	1.76	nd	-	0.88	0.67	0.85	0.59	nd	-	0.17	0.20	0.21	0.24	nd	-	1.34	0.02	1.17	0.14	nd	-	0.13	0.01	0.26	0.24	nd	-
3	sec-Butyl acetate	A	959	0.28	0.28	nd	-	nd	-	0.09	0.03	nd	-	nd	-	0.16	0.21	nd	-	nd	-	0.07	0.05	nd	-	nd	-	0.03	0.00	nd	-	nd	-
4	Isobutyl acetate [a]	A	983	6.46	4.60	3.66	2.98	0.51	0.15	4.45	2.12	2.48	1.02	0.71	0.54	2.00	0.20	0.97	0.27	0.22	0.00	6.63	0.96	3.30	0.13	2.36	1.78	1.57	0.02	1.25	0.56	1.03	0.22
5	2-Methylbutyl acetate [a]	A	1091	8.69	6.46	9.82	8.57	2.37	1.02	5.59	0.60	6.36	1.05	2.19	1.06	3.36	0.25	3.32	0.42	1.05	0.09	6.28	0.38	6.66	0.62	6.55	5.10	2.46	0.03	2.91	0.74	4.99	0.08
6	Isoamyl acetate[a]	A	1091	40.1	35.8	73.6	79.6	6.37	4.52	21.2	5.34	33.7	11.6	4.53	3.16	10.3	3.35	14.8	5.99	1.63	0.41	29.9	6.94	41.9	0.12	19.8	18.49	8.44	0.13	13.3	9.05	8.81	4.64
7	Hexyl acetate	A	1253	0.54	0.72	1.82	2.51	nd	-	0.23	0.13	0.34	0.09	nd	-	0.09	0.12	0.12	0.15	nd	-	0.16	0.03	0.25	0.04	nd	-	0.03	0.00	0.04	0.01	nd	-
8	Butyl acetate	B[54, 56]	1044	nd	-	1.81	2.12	nd	-	nd	-	0.60	0.27	nd	-	nd	-	0.40	0.42	nd	-	nd	-	0.78	0.02	nd	-	nd	-	0.19	0.19	nd	-
9	3-Ethoxypropyl acetate	C	1348	0.07	0.03	1.76	2.00	nd	-	0.07	0.03	1.04	0.89	nd	-	0.10	0.04	0.96	0.56	nd	-	0.09	0.00	1.62	0.08	nd	-	0.03	0.00	0.46	0.46	nd	-
10	2,3-Butanediol diacetate	B[54]	1477	0.14	0.04	1.05	1.12	nd	-	0.17	0.08	1.25	0.91	nd	-	0.28	0.14	1.62	0.94	nd	-	0.19	0.04	1.38	0.74	nd	-	0.11	0.01	0.46	0.53	nd	-
11	1,3-Propanediol, diacetate[a]	B[54]	1654	0.14	0.01	3.81	3.60	0.23	0.02	0.10	0.06	1.18	0.86	0.12	0.05	0.22	0.20	1.79	1.61	0.21	0.15	0.11	0.06	1.49	0.43	0.30	0.21	0.04	0.01	0.56	0.63	0.29	0.12
12	2,3-Dihydroxypro-pyl acetate	B[54]	2282	nd	-	0.51	0.43	0.34	0.04	nd	-	0.19	0.10	0.02	0.00	nd	-	0.24	0.06	0.78	0.20	nd	-	0.27	0.01	0.12	0.13	nd	-	0.08	0.03	0.06	0.01
13	Benzyl acetate[a]	A	1720	0.12	0.05	1.22	1.36	0.10	0.02	0.20	0.10	1.25	0.85	0.16	0.07	0.12	0.07	0.64	0.42	0.12	0.05	0.19	0.11	1.31	0.76	0.37	0.23	0.18	0.02	0.90	1.17	0.96	1.17
14	2-Phenylethyl acetate[a]	A	1810	5.19	2.55	22.2	25.2	4.78	1.12	6.28	2.67	15.8	9.42	5.73	2.14	5.92	1.77	12.6	4.95	5.60	0.57	6.74	2.02	18.3	5.92	16.3	12.7	2.63	0.22	4.98	3.36	14.6	5.51
15	Methyl acetate	B[54]	874	1.04	0.11	nd	-	nd	-	0.92	0.21	nd	-	nd	-	0.97	0.08	nd	-	nd	-	1.18	0.16	nd	-	nd	-	0.55	0.00	nd	-	nd	-

Total of acetic esters			112	60.2	148	152	14.7	6.90	82.7	12.6	80.9	35.6	13.5	7.03	53.1	28.1	40.7	19.7	9.60	1.47	107	28.7	99.1	9.91	45.8	38.7	55.2	1.18	34.0	19.9	30.75	11.7
ACIDS																																
16	Acetic acid[a]	A	1437	9.27	0.00	40.1	9.85	3.26	0.62	10.4	7.89	55.4	37.7	4.24	3.54	14.02	1.25	25.7	9.38	4.94	4.42	14.1	2.63	26.2	13.2	12.3	7.16	8.90	0.32	20.7	2.79	9.51	6.38
17	Propanoic acid	A	1518	nd	-	2.19	1.37	0.37	0.09	nd	-	2.65	0.80	0.27	0.10	nd	-	3.12	2.39	0.32	0.16	nd	-	2.28	1.32	0.59	0.35	nd	-	1.09	0.81	0.65	0.30
18	Isobutyric acid[a]	A	1564	0.32	0.04	3.02	0.94	0.56	0.08	0.39	0.31	5.82	3.01	0.58	0.29	0.35	0.01	4.32	1.19	0.40	0.02	0.43	0.26	5.56	3.37	1.03	0.34	0.16	0.00	2.08	0.80	0.81	0.13
19	Butanoic acid [a]	A	1629	0.11	0.03	10.30	7.86	0.53	0.08	0.16	0.19	13.9	12.4	0.52	0.40	0.21	0.20	11.5	12.7	0.55	0.42	0.19	0.15	13.2	13.5	1.25	0.63	0.05	0.00	1.91	0.92	1.08	0.04
20	Isovaleric acid[a]	A	1669	2.93	0.59	24.4	16.7	3.95	0.52	3.84	2.03	27.6	6.79	4.87	0.94	4.64	0.70	18.9	5.14	5.01	0.99	4.04	1.75	26.4	8.53	10.1	6.43	2.60	0.01	13.27	4.66	14.6	3.38
21	2-methylbutanoic acid	A	1539	nd	-	nd	-	0.48	0.04	nd	-	nd	-	0.57	0.26	nd	-	nd	-	0.45	0.08	nd	-	nd	-	0.99	0.34	nd	-	nd	-	0.92	0.21
22	Pentanoic acid	A	1722	nd	-	0.21	0.20	0.08	0.01	nd	-	0.34	0.17	0.13	0.05	nd	-	0.26	0.22	0.08	0.01	nd	-	0.33	0.19	0.31	0.22	nd	-	0.09	0.05	0.27	0.03
23	Hexanoic acid[a]	A	1855	0.07	0.01	3.28	3.42	0.73	0.17	0.11	0.08	3.84	1.02	0.71	0.18	0.12	0.01	2.49	0.73	0.64	0.08	0.14	0.04	3.43	1.60	1.92	1.53	0.08	0.00	1.22	0.67	1.97	0.56
24	2-Ethylhexanoic acid[a]	B^{1}	1958	0.02	0.01	0.21	0.23	0.08	0.02	0.07	0.01	0.27	0.21	0.12	0.06	0.03	0.01	0.11	0.06	0.06	0.01	0.05	0.04	0.31	0.16	0.25	0.13	0.01	0.00	0.04	0.01	0.15	0.01
25	Octanoic acid[a]	A	2084	0.12	0.03	3.38	3.57	1.87	0.28	0.17	0.12	3.07	1.20	1.99	0.46	0.11	0.03	2.21	1.13	2.26	0.27	0.15	0.14	2.21	0.02	4.03	2.44	0.07	0.00	1.51	0.88	6.49	1.13
26	Sorbic Acid	$B^{1,5}_{5}$	2138	nd	-	0.03	0.04	0.20	0.09	nd	-	0.12	0.17	0.04	0.02	nd	-	0.04	0.05	0.09	0.02	nd	-	0.00	0.00	0.09	0.04	nd	-	0.03	0.04	0.11	0.07
27	Nonanoic acid	A	2163	nd	-	0.30	0.32	0.40	0.11	nd	-	0.22	0.10	0.46	0.06	nd	-	0.16	0.07	0.55	0.03	nd	-	0.16	0.01	1.39	1.25	nd	-	0.12	0.05	2.23	0.22
28	Decanoic acid[a]	A	2298	0.19	0.03	10.7	10.2	5.28	1.53	0.15	0.12	7.38	6.03	4.89	0.99	0.18	0.09	5.75	2.54	5.17	1.12	0.14	0.13	4.00	1.25	8.75	4.47	0.10	0.02	3.91	1.72	13.1	6.81
29	Dodecanoic acid	B^{1}	2456	nd	-	0.17	0.17	0.33	0.12	nd	-	0.24	0.31	0.26	0.12	nd	-	0.13	0.08	0.32	0.11	nd	-	0.03	0.00	0.71	0.51	nd	-	0.07	0.06	2.83	2.27
30	Tetradecanoic acid	$B^{1,2}$	$2^{6}0$	nd	-	0.22	0.22	nd	-	nd	-	0.28	0.30	nd	-	nd	-	0.18	0.10	nd	-	nd	-	0.05	0.02	nd	-	nd	-	0.08	0.08	nd	-
31	Pentadecanoic acid	$B^{1,2}$	2670	nd	-	0.08	0.09	nd	-	nd	-	0.13	0.13	nd	-	nd	-	0.07	0.04	nd	-	nd	-	0.03	0.02	nd	-	nd	-	0.03	0.03	nd	-
32	Hexadecanoic acid	$B^{1,2}$	2776	nd	-	0.30	0.28	nd	-	nd	-	0.51	0.33	nd	-	nd	-	0.22	0.11	nd	-	nd	-	0.20	0.11	nd	-	nd	-	0.12	0.08	nd	-
Total of acids				13.04	0.75	98.9	$^{2}.6$	18.1	3.70	15.3	10.7	121	70.6	19.6	7.5	19.6	2.31	75.3	35.9	20.8	7.73	19.2	5.14	84.4	43.3	43.7	25.8	11.9	0.36	46.3	13.6	$^{1}.7$	21.5
ALCOHOLS																																
33	Ethanol	A	932	5.88	4.62	nd	-	nd	-	5.79	2.42	nd	-	nd	-	1.36	1.24	nd	-	nd	-	6.81	0.97	nd	-	nd	-	3.28	0.14	nd	-	nd	-
34	Isoamyl alcohol	A	1207	7.90	8.08	21.7	23.6	nd	-	4.48	1.99	8.00	$3.^{1}$	nd	-	2.14	0.66	3.47	1.70	nd	-	5.67	0.30	10.6	0.15	nd	-	$2.^{4}$	0.07	5.00	4.68	nd	-

35	2-Methy-1-butanol[a]	A	1207	4.77	2.98	7.44	6.19	1.83	0.97	3.38	0.96	4.62	1.02	1.42	0.[4]	2.06	0.09	2.[1]	0.50	0.74	0.00	3.[2]	0.72	4.85	0.70	3.49	2.36	2.14	0.06	3.00	1.68	3.93	1.65
36	2-Ethyl-1-hexanol	A	1489	0.12	0.05	nd	-	nd	-	0.16	0.01	nd	-	nd	-	0.08	0.04	nd	-	nd	-	0.15	0.04	nd	-	nd	-	0.14	0.00	nd	-	nd	-
37	2-Phenylethanol[a]	A	1930	1.[1]	0.[5]	33.3	37.4	3.77	1.34	1.85	0.63	20.1	9.80	3.39	1.07	1.60	0.34	15.0	5.19	3.[3]	0.01	1.76	0.75	22.1	6.94	10.2	8.50	1.17	0.20	10.8	8.80	15.0	10.4
38	1-Hexanol	A	1348	nd	-	5.71	7.84	nd	-	nd	-	1.36	0.38	nd	-	nd	-	0.[2]	0.71	nd	-	nd	-	0.93	0.22	nd	-	nd	-	0.19	0.09	nd	-
39	1,3-Propanediol	B[1]	18[6]	nd	-	0.09	0.06	nd	-	nd	-	0.13	0.11	nd	-	nd	-	0.04	0.01	nd	-	nd	-	0.14	0.09	nd	-	nd	-	0.04	0.01	nd	-
40	2-Furanmethanol	A	1669	0.41	0.14	nd	-	nd	-	0.46	0.07	nd	-	nd	-	0.29	0.13	nd	-	nd	-	0.38	0.06	nd	-	nd	-	0.25	0.07	nd	-	nd	-
Total of alcohols				20.6	16.4	68.2	75.0	5.61	2.31	16.1	6.06	34.2	14.8	4.81	1.65	7.53	2.50	21.6	8.11	4.30	0.02	18.3	2.83	38.6	8.10	13.6	10.9	9.[2]	0.53	19.0	15.3	18.9	12.0
ALDEHYDES																																	
41	Benzaldehyde [a]	A	1514	0.87	1.13	21.0	28.8	0.51	0.[5]	0.47	0.02	5.81	0.96	0.36	0.08	1.17	1.17	10.8	11.2	0.68	0.[2]	0.29	0.23	3.71	3.92	0.45	0.06	0.22	0.00	1.61	0.66	0.93	0.01
42	Furfural	A	1437	1.14	0.79	32.6	39.7	nd	-	1.53	0.89	29.6	12.9	nd	-	1.00	0.81	16.6	17.4	nd	-	0.75	0.21	14.2	8.90	nd	-	0.80	0.05	10.4	7.52	nd	-
43	5-Methylfurfural[a]	A	1[3]4	0.08	0.05	1.08	1.33	0.04	0.04	0.11	0.09	1.05	0.62	0.07	0.03	0.06	0.06	0.53	0.62	0.03	0.03	0.06	0.01	0.[5]	0.05	0.10	0.08	0.10	0.00	0.76	0.27	0.37	0.23
44	5-Acetoxymethyl-2-furaldehyde	B[1]	2191	nd	-	0.02	0.02	nd	-	nd	-	0.04	0.03	nd	-	nd	-	0.01	0.01	nd	-	nd	-	0.04	0.03	nd	-	nd	-	0.07	0.02	nd	-
45	5-Hydroxymethylfurfural	A	2480	nd	-	0.06	0.03	0.10	0.03	nd	-	0.10	0.00	0.19	0.01	nd	-	0.02	0.01	0.06	0.01	nd	-	0.14	0.05	0.50	0.42	nd	-	0.14	0.06	1.10	0.04
Total of aldehydes				2.09	1.97	[1].78	69.83	0.66	0.65	2.12	1.00	36.6	14.5	0.62	0.12	2.23	2.04	27.9	29.2	0.76	0.[6]	1.10	0.45	18.6	12.9	1.05	0.[3]	1.13	0.06	12.9	8.53	2.40	0.28
ETHYL ESTERS																																	
46	Ethyl propanoate	A	932	1.[3]	1.42	1.69	2.02	nd	-	0.70	0.43	0.[2]	0.32	nd	-	0.29	0.38	0.19	0.26	nd	-	0.79	0.29	0.[5]	0.28	nd	-	0.21	0.01	0.28	0.21	nd	-
47	Ethyl isobutyrate	A	947	1.89	1.64	0.78	0.75	nd	-	1.11	0.64	0.45	0.18	nd	-	0.18	0.21	0.10	0.11	nd	-	1.28	0.39	0.43	0.21	nd	-	0.24	0.00	0.15	0.04	nd	-
48	Ethyl butyrate [a]	A	999	1.50	1.34	4.[5]	5.33	0.14	0.07	0.88	0.90	1.66	1.[3]	0.18	0.21	0.32	0.45	0.[6]	0.83	0.04	0.06	0.97	0.79	1.67	1.[3]	0.30	0.04	0.09	0.00	0.23	0.09	0.07	0.02
49	Ethyl 2-methylbutyrat[a]	A	1014	1.16	0.94	0.[1]	0.44	0.09	0.03	0.61	0.22	0.26	0.11	0.10	0.08	0.10	0.11	0.04	0.05	0.01	0.01	0.69	0.09	0.30	0.07	0.26	0.15	0.13	0.00	0.07	0.00	0.09	0.04
50	Ethyl isovalerate[a]	A	1036	8.89	6.96	6.21	5.04	1.05	0.47	6.19	0.53	4.75	0.60	1.15	0.[6]	0.91	1.10	0.65	0.81	0.12	0.13	6.40	0.24	4.62	0.79	2.98	2.00	1.80	0.03	1.[5]	0.03	1.65	0.52
51	Ethyl pentanoate	A	1093	nd	-	0.53	0.62	nd	-	nd	-	0.22	0.11	nd	-	nd	-	0.08	0.06	nd	-	nd	-	0.27	0.05	nd	-	nd	-	0.07	0.04	nd	-

52	Ethyl hexanoate[a]	A	1207	1.[4]	1.84	9.12	12.33	0.34	0.33	0.52	0.15	1.32	0.86	0.16	0.11	0.10	0.11	0.17	0.20	0.02	0.03	0.72	0.13	1.65	0.39	0.65	0.[6]	0.15	0.00	0.23	0.15	0.24	0.03
53	Ethyl 3-ethoxypropanoate	B[1,2]	1326	nd	-	0.80	1.05	nd	-	nd	-	0.24	0.18	nd	-	nd	-	0.13	0.14	nd	-	nd	-	0.27	0.14	nd	-	nd	-	0.06	0.06	nd	-
1	Ethyl heptanoate	A	1319	0.04	0.05	nd	-	nd	-	0.01	0.01	nd	-	nd	-	0.00	0.00	nd	-	nd	-	0.02	0.01	nd	-	nd	-	0.00	0.00	nd	-	nd	-
2	Ethyl octanoate	A	1437	1.47	1.91	13.52	18.40	nd	-	0.44	0.01	1.[5]	0.66	nd	-	0.06	0.06	0.26	0.24	nd	-	0.44	0.00	1.65	0.[2]	nd	-	0.15	0.00	0.43	0.23	nd	-
3	Ethyl sorbate	B[1]	1491	0.21	0.23	2.23	3.07	nd	-	0.04	0.01	0.04	0.01	nd	-	0.04	0.03	0.06	0.05	nd	-	0.04	0.00	0.04	0.00	nd	-	0.03	0.00	0.05	0.05	nd	-
4	Ethyl decanoate	A	1629	0.22	0.28	nd	-	0.23	0.19	0.07	0.04	nd	-	0.12	0.01	0.01	0.01	nd	-	0.01	0.01	0.04	0.01	nd	-	0.17	0.05	0.05	0.00	nd	-	0.23	0.02
5	Ethyl benzoate	A	16[1]	0.13	0.10	nd	-	0.09	0.04	0.07	0.03	nd	-	0.06	0.03	0.04	0.03	nd	-	0.03	0.02	0.09	0.01	nd	-	0.21	0.18	0.05	0.00	nd	-	0.25	0.17
6	Diethyl succinate[a]	A	1669	0.81	0.99	24.3	33.9	0.71	0.85	0.32	0.34	2.42	2.78	0.22	0.22	0.07	0.04	0.44	0.37	0.06	0.03	0.34	0.31	2.69	2.39	0.52	0.21	0.07	0.01	0.61	0.77	0.40	0.41
60	Ethyl phenyl acetate[a]	A	1779	1.18	0.84	11.02	13.33	1.02	0.43	1.19	0.79	6.11	4.81	1.02	0.[6]	0.20	0.21	0.98	1.11	0.24	0.23	1.14	0.87	6.17	4.72	2.13	0.99	0.21	0.02	0.83	0.[2]	1.44	0.36
61	Ethyl dodecanoate	A	1818	0.01	0.00	0.05	0.07	nd	-	0.01	0.00	0.01	0.00	nd	-	0.01	0.01	0.00	0.00	nd	-	0.01	0.01	0.01	0.00	nd	-	0.03	0.00	0.02	0.02	nd	-
62	Diethyl malate	B[2]	2038	nd	-	0.07	0.10	nd	-	nd	-	0.01	0.01	nd	-	nd	-	0.00	0.00	nd	-	nd	-	0.01	0.00	nd	-	nd	-	0.00	0.00	nd	-
63	Methyl hexadecanoate	B[1]	2222	0.05	0.02	nd	-	nd	-	0.09	0.01	nd	-	nd	-	0.07	0.06	nd	-	nd	-	0.05	0.03	nd	-	nd	-	0.06	0.03	nd	-	nd	-
64	Ethyl hexadecanoate	A	2260	0.02	0.01	nd	-	nd	-	0.01	0.01	nd	-	nd	-	0.01	0.00	nd	-	nd	-	0.01	0.01	nd	-	nd	-	0.04	0.00	nd	-	nd	-
65	Ethyl furoate[a]	A	1608	0.10	0.05	1.41	1.63	0.05	0.01	0.08	0.03	0.74	0.40	0.05	0.02	0.03	0.01	0.23	0.12	0.02	0.01	0.09	0.02	0.86	0.23	0.15	0.12	0.04	0.00	0.25	0.28	0.13	0.11
66	Ethyl hydrogen succinate	B[4]	22[1]	nd	-	nd	-	0.21	0.20	nd	-	nd	-	0.11	0.08	nd	-	nd	-	0.05	0.02	nd	-	nd	-	0.18	0.02	nd	-	nd	-	0.08	0.10
Total of ethyl esters				20.8	18.6	76.8	98.1	3.92	2.63	12.3	4.15	20.4	12.6	3.17	1.94	2.43	2.83	3.92	4.34	0.61	0.[1]	13.1	3.21	21.2	11.4	7.[4]	4.35	3.37	0.13	4.86	2.52	4.60	1.78
KETONES																																		
67	2,3-Butanedione	C	947	1.[1]	1.26	nd	-	nd	-	1.23	0.27	nd	-	nd	-	2.[6]	0.76	nd	-	nd	-	1.40	0.02	nd	-	nd	-	0.86	0.01	nd	-	nd	-
68	Methyl Isobutyl ketone	B[1,2]	983	0.20	0.16	nd	-	nd	-	0.37	0.38	nd	-	nd	-	0.04	0.01	nd	-	nd	-	0.09	0.02	nd	-	nd	-	0.03	0.00	nd	-	nd	-
69	Acetoin	A	1275	0.[5]	0.39	5.27	4.80	nd	-	0.90	0.41	4.16	2.01	nd	-	2.09	1.61	6.61	3.98	nd	-	1.06	0.18	5.68	0.14	nd	-	0.74	0.01	5.25	5.63	nd	-
70	1-Hydroxy-2-propanone	A	1279	1.50	0.35	nd	-	nd	-	1.[3]	0.15	nd	-	nd	-	1.[5]	1.04	nd	-	nd	-	1.94	0.38	nd	-	nd	-	1.42	0.26	nd	-	nd	-

71	3-Nonanone	A	1348	0.06	0.06	nd	-	nd	-	0.02	0.01	nd	-	nd	-	0.01	0.01	nd	-	nd	-	0.02	0.00	nd	-	nd	-	0.01	0.00	nd	-	nd	-
72	2-Acetoxy-3-butanone	B[1]	1370	0.14	0.06	nd	-	nd	-	0.23	0.14	nd	-	nd	-	0.[2]	0.30	nd	-	nd	-	0.29	0.06	nd	-	nd	-	0.20	0.01	nd	-	nd	-
73	2-Acethylfuran	A	1498	0.10	0.04	1.60	1.71	nd	-	0.12	0.04	1.70	0.21	nd	-	0.09	0.04	0.78	0.[5]	nd	-	0.11	0.03	1.70	0.20	nd	-	0.12	0.00	1.39	0.62	nd	-
74	2(5H)-Furanone	B[5]	17[4]	0.23	0.07	nd	-	nd	-	0.26	0.03	nd	-	nd	-	0.19	0.08	nd	-	nd	-	0.23	0.00	nd	-	nd	-	0.17	0.02	nd	-	nd	-
Total of ketones				4.33	2.40	6.87	6.51	0.00	0.00	4.69	1.45	5.86	2.22	0.00	0.00	7.13	3.85	7.39	4.[3]	0.00	0.00	5.14	0.70	7.38	0.34	0.00	0.00	3.[2]	0.32	6.64	6.25	0.00	0.00
OTHERS																																		
75	Trans-linalool oxide	B[6]	1460	0.09	0.04	nd	-	nd	-	0.08	0.02	nd	-	nd	-	0.05	0.02	nd	-	nd	-	0.06	0.01	nd	-	nd	-	0.12	0.00	nd	-	nd	-
76	TDN[a]	B[2]	1732	0.17	0.14	0.35	0.25	0.13	0.01	0.09	0.03	0.12	0.07	0.06	0.01	0.13	0.17	0.13	0.15	0.10	0.13	0.06	0.01	0.05	0.02	0.11	0.08	0.70	0.00	0.41	0.[1]	2.32	3.05
77	2,4,5-Trimethyl-1,3-Dioxolane	B[2]	932	2.82	2.41	2.38	2.03	nd	-	1.04	0.63	0.62	0.33	nd	-	0.77	0.51	0.39	0.32	nd	-	1.17	0.44	0.66	0.28	nd	-	0.41	0.04	0.25	0.29	nd	-
78	1,4:3,6-Dianhydro-α-d-glucopyranose	C	2433	0.06	0.01	0.02	0.02	nd	-	0.08	0.04	0.05	0.05	nd	-	0.05	0.00	0.01	0.00	nd	-	0.06	0.00	0.07	0.02	nd	-	0.05	0.00	0.01	0.01	nd	-
79	Methyl salicylate[a]	A	1765	0.02	0.00	0.11	0.11	0.02	0.00	0.08	0.04	0.41	0.29	0.08	0.05	0.02	0.01	0.07	0.04	0.02	0.01	0.07	0.06	0.37	0.35	0.13	0.02	0.01	0.00	0.03	0.01	0.08	0.01
80	Isopropyl myristate	B[1]	2041	0.02	0.01	nd	-	nd	-	0.02	0.01	nd	-	nd	-	0.07	0.04	nd	-	nd	-	0.05	0.02	nd	-	nd	-	0.10	0.08	nd	-	nd	-
81	4-Ethylguaiacol	A	2041	0.03	0.01	0.25	0.27	nd	-	0.07	0.01	0.36	0.09	nd	-	0.03	0.01	0.15	0.06	nd	-	0.06	0.02	0.36	0.09	nd	-	0.02	0.00	0.07	0.05	nd	-
Total of others				3.20	2.61	3.11	2.68	0.15	0.01	1.47	0.77	1.[3]	0.82	0.14	0.05	1.12	0.77	0.75	0.[4]	0.12	0.13	1.52	0.[4]	1.51	0.76	0.24	0.10	1.41	0.14	0.78	0.89	2.40	3.06

[a] Volatile compounds extracted in common by the three sampling methods.

[b] [b]ID (identification): reliability of identification: A, mass spectrum and LRI agreed with standards; B, mass spectrum agreed with mass spectral data base and LRI agreed with the literature data obtained with standards; C, mass spectrum agreed with mass spectral data base. Reference which matched the experimental LRI: 1: National Center for Biotechnology Information (2005); 2: Morales et al. (2017); 3: Ruiz-Bejarano et al. (2013); 4: Selli et al. (2004); 5: Pozo-Bayón et al. (2007); 6: Loscos et al. (2007).

[c] RI: Retention Index.

[d] ARm: Mean relative areas.

nd: peak not detected

ARTÍCULO 9

A comparative study of the volatile profile of wine vinegars with protected designation of origin by headspace stir bar sorptive extraction

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A comparative study of the volatile profile of wine vinegars with protected designation of origin by headspace stir bar sorptive extraction

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ABSTRACT

The characteristic volatile profile of the Spanish *Vinagre de Jerez* (VJ), *Vinagre de Condado de Huelva* (VC) and *Vinagre de Montilla-Moriles* (VMM) protected designation of origin (PDO) wine vinegars has been studied and compared for the first time by headspace stir bar sorptive extraction-gas chromatography–mass spectrometry (HSSE-GC–MS). The possible markers of each category and PDO were assessed. Acetates were the majority group in all vinegars, while ketones, C_{13}-norisoprenoids and volatile phenols showed significant differences between the three PDOs. Analysis of variance (ANOVA), heatmap and partial least squares-discriminant analysis (PLS-DA) were performed. According to these results, 1-heptanol, methyl nonanoate, 2-methylbutanoic acid, 2,2,6-trimethyl-cyclohexanone, *trans*-2-decenal, eucalyptol and α-terpineol, were the most significant compounds for differentiating of VC, diacetyl and acetoin, ethyl 3-ethoxypropanoate, 2- and 3-heptanone, 2-methyl-1-hexadecanol, 1-octen-3-ol, *p*-Cresol and camphene for VMM; and β-damascenone, 5-hydroxymethylfurfural, 3-heptanol, *trans*-2-hexen-1-ol and *trans*-2-hexen-1-yl acetate for VJ. Classification results showed that 100% of PDO samples were correctly classified, reaffirming the utility of the volatile profiles for classifying and authenticating wine vinegar PDOs.

1. Introduction

Wine vinegar is produced by microorganisms performing two different biochemical processes. The first process is an alcoholic fermentation in which natural sugars are converted to ethanol. The second fermentation is acetous, in which ethanol is transformed into acetic acid by the action of acetic acid bacteria (Tesfaye et al., 2010). For many years, wine vinegar was regarded as a low-value secondary product. However, it has now become a highly-demanded product, being both a valued food product in gastronomy and a condiment (Ríos-Reina et al., 2017). This recent added value, together with the high quality of some wine vinegars linked to geographical origin and produced by traditional methods, have led the European Union (EU) to incorporate some such vinegars within the Protected Designation of Origin (PDO) regulatory system (Council Regulation (EC) No 510/2006). This regulatory system also provides protection for consumers against falsifications and guarantees the high quality of PDO wine vinegars.

Nowadays, Spain is one of the major producers of wine vinegars, being some of these vinegars, with high quality, registered with a PDO: *Vinagre de Jerez*, *Vinagre de Montilla-Moriles* and *Vinagre de Condado de Huelva*. These wine vinegars come from specific areas in the south of Spain, and are produced by traditional techniques from the wines that are also protected under the corresponding PDO and produced in the same geographical area. Furthermore, within each PDO, there are different categories according to their time and type of ageing in wooden barrels, as well as other characteristics of production.

The regulations applicable to the *Vinagre de Jerez* and *Vinagre de Montilla-Moriles* PDOs (BOJA, 2008a; BOJA, 2008c) describe three categories according to their dynamic ageing time in oak barrels under the system known as *Criaderas y solera:* the commonly-called *Crianza* is aged in wood for at least 6 months; the *Reserva* category has a minimum ageing time of 2 years and *Gran Reserva* is aged for 10 years or more. These regulations also include two semi-sweet categories: *Vinagre al Pedro Ximénez* and *Vinagre al Moscatel*.

The *Vinagre Condado de Huelva* PDO regulation (BOJA, 2008b) also establishes categories according to ageing by the *Criaderas y solera* system. This PDO establishes a non-aged category followed by *Solera*, which is aged for at least 6 months, and *Reserva*, aged for at least 2 years. Furthermore, this PDO has an additional category, *Añada*, where the vinegar is statically aged in wooden barrels for at least

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3 years.

The age of the vinegars and their semi-sweet property has a remarkable impact on the vinegars' aroma and is the reason for their higher price regarding those vinegars without a PDO. Aroma is, therefore, one of the main quality indicators of vinegars and it depends on the volatile compounds profile. The characteristic volatile profile of a vinegar is determined by different factors: the raw material used, the production process and the ageing in wooden barrels (Callejón, Morales, Silva Ferreira, & Troncoso, 2008; Chinnici et al., 2009). Regarding each PDO, some of these parameters are different. Thus, the raw material used (i.e. the grape for the raw wine) for *Vinagre de Jerez* PDO is manly the *Palomino* grape variety, while for *Vinagre de Condado de Huelva* PDO and *Vinagre de Montilla-Moriles* PDO are *Zalema* and *Pedro Ximénez* grape varieties, respectively. The production process also varies from one PDO to another. Thus, although in general, wine vinegars are made by the same system (i.e. criaderas and solera), some differences between the wood of barrels and also in the production process of the sweet categories are founded. Finally, during the ageing process in wooden barrels, the volatile profile is enriched by a process of concentration. This results from a moderate loss of water through the pores of the wood. In addition, during the ageing process, some compounds are transferred from the wood to the vinegar and new compounds are created by the chemical reactions that take place. All of these ageing processes have a great influence on the aromatic composition of the final wine vinegar, resulting in high-quality vinegars with excellent organoleptic characteristics (Callejón, Morales, et al., 2008).

The importance of volatile compounds has raised interest in determining them in order to obtain objective information on vinegar aroma. Gas chromatography-mass spectrometry (GC–MS) has been the most widely-used technique for analysing volatile compounds in vinegars (Callejón, Torija, Mas, Morales, & Troncoso, 2010; Chinnici et al., 2009; Durán-Guerrero, Chinnici, Natali, & Riponi, 2015; Pizarro, Esteban-Díez, Sáenz-González, & González-Sáiz, 2008). To perform these analyses accurately, a prior extraction is required in order to concentrate the compounds. To analyse the volatile composition of wine vinegars, different extraction techniques have been applied, including dynamic headspace sampling (DHS) (Manzini et al., 2011; Ríos-Reina, Morales, García-González, Amigo, & Callejón, 2018), solid phase microextraction (SPME) (Natera Marín, Castro Mejías, de Valme García Moreno, García Rowe, & García Barroso, 2002), or stir bar sorptive extraction in immersion (SBSE) (Guerrero, Marín, Mejías, & Barroso, 2006) and headspace sampling (HSSE) (Callejón, González, Troncoso, & Morales, 2008; Ríos-Reina et al., 2018). According to the results obtained in our previous work comparing different extraction techniques (Ríos-Reina et al., 2018), HSSE provided the greatest knowledge of the volatile profile in these vinegars. This technique is able to obtain the greatest number of compounds for most of the chemical families in wine vinegar. HSSE is based on the sorption of analytes on a film of polydimethylsiloxane (PDMS) coated onto the magnet of a stir bar named Twister incorporated into a special device placed in the headspace of a glass vial (Bicchi, Iori, Rubiolo, & Sandra, 2002). The extraction of volatile compounds by HSSE sampling has several advantages such as low contamination risk, a high analyte recovery rate and an increase in the stir bar and fibre's lifetime (Ríos-Reina et al., 2018).

A wine vinegar's volatile profile obtained by HSSE-GC–MS analysis is a complex dataset and its treatment is time-consuming. Nowadays, many authors perform the data treatment by Multivariate Curve Resolution (MCR) because it helps to solve some problems such as baseline drifts, co-elution and overlapping peaks, as well as transforming complex data into a simple model of pure responses (Amigo, Skov, Bro, Coello, & Maspoch, 2008; Ríos-Reina et al., 2018).

The combination of growing consumer demand, the increasing diversity of wine vinegars, and the high quality of these PDO wine vinegars have created the need to characterise them and to establish an adequate quality control in order both to defend their identity and to combat fraud (Ríos-Reina, Callejón, Oliver-Pozo, Amigo, & García-González, 2017; Ríos-Reina, Elcoroaristizabal, et al., 2017) being the volatile profile a suitable tool for it. Among these PDO high-quality wine vinegars, the volatile profile of *Vinagre de Montilla-Moriles* and *Vinagre de Condado de Huelva PDOs* have only been studied in our previous work (Ríos-Reina et al., 2018) in which not all the categories were considered (i.e. a few *Reserva* samples were studied) since the aim was to compare different extraction techniques.In addition, there is a lack of comparative studies among these three PDOs by using the same extraction technique and analytical method, which assert reliable and robust data that enable a suitable characterisation, comparison and differentiation between wine vinegar PDOs.

In this context, once HSSE was selected as the most suitable extraction method (Ríos-Reina et al., 2018), the objective of this work was three-fold: firstly, to characterise and compare, for the first time, the volatile profile of each Spanish PDO wine vinegar and their corresponding categories (aged and sweet); secondly, to differentiate and classify through volatile profiles the three PDO wine vinegars; and thirdly, to determine the volatile compounds that could be considered possible markers of authenticity of these PDO wine vinegars.

2. Materials and methods

2.1. Samples

A total of fifty wine vinegars from the three Spanish PDOs were analysed in this study and groped as follows: 20 wine vinegars belonged to *Vinagre de Jerez* PDO, coded as VJ, being grouped in 3 different categories (7 *Crianza*, 7 *Reserva* and semi-sweet category 6 *Pedro Ximénez*); 17 wine vinegars belonging to *Vinagre de Condado de Huelva* PDO, coded as VC, grouped in 3 categories (5 samples *without ageing*, 4 *Solera* and 8 *Reserva*); and 13 wine vinegars belonging to *Vinagre de Montilla-Moriles* PDO, coded as VMM, and grouped in three categories (6 *Crianza*, 2 *Reserva* and 5 *Pedro Ximénez*). All these samples were provided by different wineries through their corresponding Regulatory Councils. More information about samples and codes are shown in Table 1.

2.2. Chemicals

The following standards of volatile compounds used for identification were bought from different commercial sources. Thus, the standards obtained from Sigma®-Aldrich (Madrid, Spain) were: 2-methylpropyl acetate (≥97%), 3-methylbutyl acetate (≥97%), 2-ethylbutyl acetate (≥98), hexyl acetate (99%), benzyl acetate (≥99.7%), benzoic acid (≥99.5%), 2-methylpropanoic acid (99%), butanoic acid (≥99%), 2-methylbutanoic acid (98%), pentanoic acid (≥99%), hexanoic acid (99.5%), 2-ethylhexanoic acid (98%), heptanoic acid (97%), octanoic

Table 1
Total samples of PDO wine vinegars analysed by HSSE-GC–MS.

Protected designation of origin	Grape variety	Code	Categories	Code	Ageing time (months)	n
"Vinagre de Jerez"	*Palomino*	VJ	"Crianza"	JCR	≥6	7
			"Reserva"	JRE	≥24	7
			"Pedro Ximénez"	JPX	–	6
"Vinagre de Condado de Huelva"	*Zalema*	VC	"Sin Crianza"	CSC	0	5
			"Solera"	CSO	≥6	4
			"Reserva"	CRE	≥12	8
"Vinagre de Montilla Moriles"	*Pedro Ximénez*	VMM	"Crianza"	MMCR	≥6	6
			"Reserva"	MMRE	≥24	2
			"Pedro Ximénez"	MMPX	–	5

acid (99%), nonanoic acid (96%), decanoic acid (96%), dodecanoic acid (99%), 1-hexanol (98%), trans-2-hexen-1-ol (96%), 1-octen-3-ol (98%), 2-Ethyl-1-hexanol (≥99.6%), benzyl alcohol (99%), octanal (99%), nonanal (95%), 2-furfuraldehyde (≥98.5%), 5-hydroxymethylfurfural (≥99%), ethyl propionate (99%), ethyl 2-methylpropanoate (98%), ethyl butanoate (99%), ethyl 3-methylpropanoate (98%), ethyl hexanoate (99%), ethyl pentanoate (99%), ethyl heptanoate (99%), ethyl octanoate (99%), ethyl benzoate (99%), ethyl 2-furoate (99%), ethyl decanoate (99%), ethyl phenylacetate (99%), ethyl hexadecanoate (99%), methyl salicylate (99%), 3-heptanone (98%), 2,6-Dimethyl-4-heptanone (99%), 2-heptanone (≥98%), 1-hydroxy-2-propanone (95%), 6-Methyl-5-hepten-2-one (99%), 3-Nonanone (99%), 2-nonanone (99%), 2-acetylfuran (99%), acetophenone (98%), benzophenone (99%), β-damascenone (≥98%), guaiacol (98%), 4-ethylguaiacol (98%), eucalyptol (99%), cis-β-Methyl-γ-octalactone (≥95%), 4-ethylphenol (99%), safranal (90%) and α-terpineol (90%).

The standards bought from Merck (Darmstadt, Germany) were: methyl acetate (99%), ethyl acetate (99.5%), propyl acetate (98%), 2-heptanol (≥99.9%), 1-heptanol (≥99%), 3-methyl-1-butanol (100%), 2-methyl-1-butanol (98%), 2-phenylethanol (100%), 1-dodecanol (98%), benzaldehyde (99%), 1-nonanol (≥98%), ethyl dodecanoate (99%), methyl decanoate (≥99.5%), diacetyl (98%), acetoin (96.0%), γ-Butyrolactone (99%), p-cresol (≥98%), eugenol (99%), and 4-Methyl-2-pentanol (99%) employed as internal standard (IS).

Finally, 2-phenylethyl acetate (≥97%), 5-methyl-2-furfuraldehyde (98%), diethyl succinate (99%), sec-butyl acetate (99%) and a series of C_{10} to C_{40} straight-chain n-alkanes (50 mg L^{-1}in n-hexane), used to calculate linear retention index (LRI), were purchased from Fluka (Madrid, Spain).

In addition, analytical-quality sodium chloride and acetic acid (≥99%) was obtained from Sigma-Aldrich, ethanol (≥99.9%) from Merck and water was obtained from a Milli-Q purification system (Millipore, USA).

2.3. Headspace sorptive extraction (HSSE)

HSSE extraction of the volatile compounds was performed following the method validated by Callejón, Morales, et al. (2008). Hence, 5 mL of wine vinegar, 1.67 g of NaCl and 10 μL of 4-methyl-2-pentanol IS solution at 1045 mg/L were placed into a special 20 mL headspace vial with open glass adapters provided by Gerstel (Müllheim and der Ruhr, Germany). A 10-mm long stir bar coated with a 0.5-mm PDMS layer (Twister, Gerstel, Müllheim an der Ruhr, Germany) was placed into the glass insert in the vial in order to perform extraction in the headspace. The vial was then tightly capped and heated in a thermostatic bath for 60 min at 62 °C. After 5 min of keeping the vial at room temperature, the stir bar was removed with tweezers, rinsed with Milli-Q water and dried with a lint-free tissue paper. Finally, the stir bar was transferred into a glass tube 60 mm long, 6 mm o.d. and 4 mm i.d. which was placed in the autosampler tray for thermal desorption and GC–MS analysis.

2.4. Gas chromatography-mass spectrometry analysis (GC–MS)

The volatile compounds were separated in a gas chromatograph Agilent 6890 GC system coupled to a quadrupole mass spectrometer Agilent 5975 inert (Agilent Technologies, Santa Clara, CA, USA) and equipped with a Gerstel Thermo Desorption System (TDS2) connected to a cryo-focusing CIS-4PTV injector (Gerstel) and an analytical J&W CPWax-57CB column (50 m*0.25 mm, 0.20-μm film thickness, Agilent Technologies, Santa Clara, CA, USA). For this study, the GC–MS conditions were reproduced from our previous works (Ríos-Reina et al., 2018; Callejón, González, et al., 2008). The stir bar extract was desorbed in splitless mode and with a 90 mL/min flow rate using helium as carrier gas. The desorption temperature program was 35 °C for 1 min, ramped at 60 °C/min to 250 °C, and held for 5 min. The CIS-4PTV injector, with a Tenax TA inlet liner, was held at −35 °C with liquid nitrogen for total desorption time and then raised to 260 °C at 10 °C/s and held for 4 min. The solvent vent mode was employed for transferring the sample to the column at 1 mL/min flow rate. Oven temperature programme was 35 °C for 5 min, being raised to 220 °C at 2.5 °C/min (held 15 min).

Mass spectra were acquired with quadrupole, source and transfer line temperatures of 150, 230 and 280 °C, respectively. Electron ionization mass spectra in the full-scan mode were recorded at 70 eV, with electron energy in the 29–300 *m/z* range. Each sample was analysed in duplicate.

As was described in our previous work (Ríos-Reina et al., 2018), all data were recorded using an MS ChemStation and were analysed by multivariate curve resolution method (MCR), using the software MATLAB v.8.5.0 (The Mathworks Inc., Natick, MA). MCR was applied to the total ion chromatogram (TIC) to obtain the peak area for each of the compounds presented in each sample and the pure mass spectral profiles for each analyte.Once the integrated peak areas were obtained, they were pre-processed by normalizing all peaks to the peak area associated with the IS (relative areas) to eliminate minor injection discrepancies between samples and extraction efficiency.

The volatile compounds were then identified based on comparing their linear retention indices (LRIs) with those of authentic reference standards and mass spectra matching to the reference mass spectra from the NIST MS Search v.2.0.. When standards were not available, those compounds which the mass spectrum agreed with mass spectral data base and LRI agreed with the literature data, were considered tentatively identified (TI). The LRIs were calculated by using the retention times of n-alkanes obtained under identical analytical conditions.

2.5. Statistical analysis

In order to study the significant differences between samples of different categories and different PDOs, three different analyses of variance (ANOVA), followed by a post hoc comparison test (Tukey's test), were performed by grouping samples into three sets: by PDO, by the categories within a PDO and by the same category across the three PDOs. These analyses were performed by the INFOSTAT software 2016 (FCA, Universidad Nacional de Córdoba, Argentina).

Partial least squares-discriminant analysis (PLS-DA) and hierarchical clustering and heatmap were constructed based on the relative areas of the extracted volatile compounds. PLS-DA was applied as a classification method using the PLS_Toolbox 7.9.5 (Eigenvector Research Inc., Wenatchee, WA) working under a MATLAB environment, in mean-centred and split data into a train and a test sets, and latent variables were assessed by the minimum classification error rate in cross-validation (venetian blind, five splits). Finally, hierarchical clustering and heatmap analyses were performed by using Pearson correlation coefficient and MetaboAnalyst 4.0 software (Xia Lab, McGill University).

3. Results and discussion

In this study, the methodology applied enabled 160 compounds in the Spanish PDO wine vinegars to be detected. The mean and standard deviations of their relative areas (RA) for each category and PDO are reported in Table I. Supplementary Material. The identified volatile compounds were grouped into fourteen groups as a function of their chemical characteristics: acetals, acetates, acids, alcohols, aldehydes, ethyl esters (EEs), methyl esters (MEs), ketones, lactones, C_{13}-norisopronoids, other esters (OEs), volatile phenols, terpenes, and finally a miscellaneous group.

Twenty-two of the 160 compounds determined here, and already reported in grapes, must and their fermentative derived products, have been described for the first time in vinegars. Although the volatile composition of the VJ PDO has been widely studied in comparison to

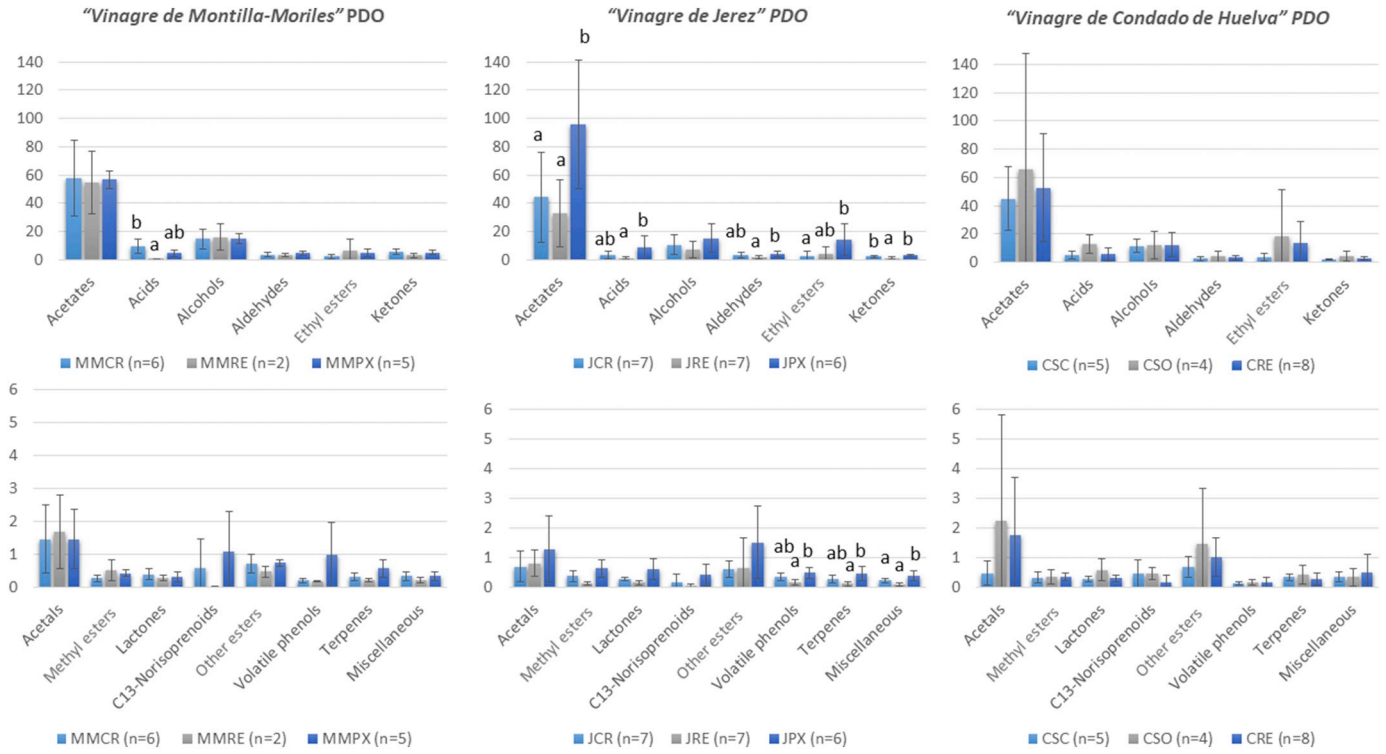

Fig. 1. Bar graph of the total values of relative areas (RA) for each chemical group according to the different categories of the three wine vinegar PDO. Codes used in the graph are listed in Table 1.

the VMM and VC PDOs (Callejón, González, et al., 2008; Castro Mejías, Natera Marín, De Valme García Moreno, & García Barroso, 2002; Cejudo-Bastante et al., 2013; Morales, Tesfaye, García-Parrilla, Casas, & Troncoso, 2002; Natera, Natera, Castro, De Valme García-Moreno, Hernández, & García-Barroso, 2003), we have, for the first time, been able to determine 7 new compounds in these kinds of vinegar, 4 of them identified by standards such as benzoic acid, methyl benzeneacetate, 6-methyl-5-hepten-2-one and *p*-cresol. Moreover, among the compounds already found in the VJ PDO, 28 compounds (21 identified by standards), had never been reported in VC and VMM. All these compounds are marked with asterisks in Table I. Supplementary Material. A similar total number of compounds was detected for each PDO: 156 in VMM, 155 in VJ and 153 in VC. The chemical groups with major numbers of compounds were ketones (24), alcohols (20), ethyl esters (19), acids (16) and acetates (15).

3.1. Characterisation and comparison of the volatile composition of the different categories included in each PDO (aged and sweet)

The total values of relative areas (RA) for each chemical group of the total amount of compounds are shown in Fig. 1. As can be seen in Fig. 1, the acetates were the group of compounds that showed the highest RA in all of the categories, especially in *Pedro Ximénez* category of VJ PDO (96 ± 45 sum of the relative areas). Among this group, the main volatile compounds, which showed the highest RA in JPX samples were methyl acetate, ethyl acetate, 2-ethylhexyl acetate (tentatively identified-TI), 3-oxobutan-2-yl acetate and benzyl acetate (Table I. Supplementary Material). Among these compounds, and due to its high RA values, ethyl acetate can be highlighted. This fact could be due to the addition of *Pedro Ximénez* wine during the production of JPX, which increases residual ethanol favouring the formation of this compound (Morales, González, Casas, & Troncoso, 2001).

The group of acids also presented significant differences in total RA values among the categories within the VMM and VJ PDOs (Fig. 1). In both cases, the *Reserva* category showed the lowest values. These results agree with the fact that during ageing, acids react with alcohols producing esters (Morales et al., 2002). VMM PDO showed significantly higher values for acids in the *Crianza* category (MMCR) than in the MMRE one, highlighting 2-methylbutanoic acid, octanoic acid and decanoic acid. In the case of VJ, the JPX category achieved the highest statistically significant values, standing out 2-methylpropanoic acid, 3-methylbutanoic acid (tentatively identified-TI) and 2-methylbutanoic acid (Table I. Supplementary Material). Regarding the rest of groups of compounds, their total values did not present significant differences between the categories of the VMM and the VC PDOs, unlike the VJ PDO (Fig. 1). Hence, aldehydes, volatile phenols and terpenes showed the same trend observed for acids in the VJ PDO, being JPX the category that achieved the highest RA values. Among them, 2-furfuraldehyde and 5-methyl-2-furfuraldehyde stood out. These compounds have been found in must from Pedro Ximénez grapes due to Maillard reactions take place during sun drying (Ruiz-Bejarano, Castro-Mejías, Rodríguez-Dodero, & García-Barroso, 2016). Finally, worthy of mention is linalool oxide, correlating to a higher level of linalool in overripe grape (Genovese, Gambuti, Piombino, & Moio, 2007; Ruiz-Bejarano, Castro-Mejías, Rodríguez-Dodero, & García-Barroso, 2013). Other significant differences were identified when comparing the ethyl esters' RA values between *Crianza* and *Pedro Ximénez* categories in VJ PDO (Fig. 1). In particular, ethyl 2-methylpropanoate, ethyl 3-methylbutanoate, ethyl hexanoate and ethyl phenylacetate, among others, were found to be at higher RA values in JPX category (Table I. Supplementary material). The possible explanation of this fact was the same above discussed, that is the increase of residual ethanol due to the addition of wine in this vinegars, that favours the formation of these kind of compounds (Morales et al., 2001). Moreover, these compounds give fruity nuances, and, as a result, could provide a greater fruity aroma in *Pedro Ximénez* vinegars (JPX). Finally, regarding the ketones, the *Reserva* samples showed lower significant values with respect to the other two categories in the three PDOs. These results did not match with results previous reported by other authors (Callejón et al., 2010) may be due to they used a control samples during different ageing times, and

Table 2
Mean, standard deviations of the relative areas (RA) of the 57 detected and identified compounds with significant differences between PDOs according to Tukey's test ($p < .05$).

			Samples													
			VMM								VJ					
Volatile compounds[1]			MMCR ($n = 6$)			MMRE ($n = 2$)			MMPX ($n = 5$)			JCR ($n = 7$)			JRE (n = 7)	
	LRI	ID[2]	RA	SD	S[3]	RA	SD	S	RA	SD	S	RA	SD	S	RA	SD
Acetals																
2-Butyl-4-methyl-1,3-dioxolane	1260	DB	0.04	0.04		0.01	0.01		0.05	0.03		0.01	0.01		0.02	0.04
Acetates																
Methyl acetate	871	STt	1.2	0.7		0.59	0.21		1.2	0.4		1.0	0.4	a,b	0.5	0.4
trans-2-Hexen-1-yl acetate	1307	M[8]	nd	nd		nd	nd	*B*	nd	nd	**B**	0.02	0.03		0.00	0.00
3-Oxobutan-2-yl acetate	1362	ST	0.7	0.4	A	0.34	0.23	*A*	0.7	0.4		0.28	0.08	b,AB	0.18	0.09
Acids																
2-Methylpropanoic acid**	1557	ST	0.31	0.20	AB	0.02	0.02		0.22	0.12		0.09	0.07	a,b,B	0.03	0.03
2-Methylbutanoic acid	1662	ST	0.43	0.19	a,B	0.03	0.02	b	0.35	0.17	a,b	0.18	0.15	b,B	0.06	0.04
(4-Hexyl-2,5-dioxo-2,5-dihydro-3-furanyl) acetic acid*	2098	DB	0.04	0.02	a,b,A	0.00	0.00	b	0.07	0.04	a,**A**	0.01	0.00	b,B	0.01	0.00
Dodecanoic acid**	2456	ST	0.02	0.01	a,A	0.01	0.00	b	0.02	0.00	a,b	0.01	0.00	B	0.01	0.01
Alcohols																
3-Methyl-1-butanol	1197	ST	5	4		4.1	2.3	*A*	3.7	1.2		2.5	1.2		1.4	1.1
3-Heptanol	1223	MS[14]	0.01	0.00	B	0.01	0.00		0.01	0.00		0.04	0.03	A	0.02	0.01
2-Heptanol*	1310	ST	nd	nd	B	nd	nd		nd	nd	**B**	0.01	0.01	a,b,A	0.01	0.01
trans-2-Hexen-1-ol	1395	ST	nd	nd		nd	nd	*B*	nd	nd	**B**	0.02	0.02		0.01	0.00
1-Octen-3-ol*	1439	ST	0.09	0.02	A	0.08	0.04	*A*	0.11	0.04	**A**	0.02	0.01	b,B	0.01	0.00
1-Heptanol**	1448	ST	nd	nd	B	nd	nd		nd	nd		nd	nd	B	nd	nd
1-Nonanol**	1655	ST	0.03	0.01		0.03	0.03	*A*	0.03	0.01		0.03	0.01	b	0.01	0.01
2-Methyl-1-hexadecanol	1749	DB	0.09	0.02	A	0.07	0.01	*A*	0.12	0.05	**A**	0.05	0.02	a,B	0.02	0.01
2-Phenylethanol**	1909	ST	2.8	1.2		3.4	1.1		2.5	0.7		2.0	0.8		1.1	0.7
Aldehydes																
Octanal**	1262	ST	0.06	0.02	B	0.04	0.01	*A*	0.07	0.02	**A**	0.02	0.01	b,B	0.01	0.00
Benzaldehyde	1503	ST	2.2	1.7		2.2	1.6		2.3	1.5		1.3	1.0		0.7	0.6
trans-2-Decenal*	1636	MS[16]	0.02	0.01	B	0.03	0.02		0.02	0.01	**B**	0.04	0.01	a,b,B	0.02	0.01
5-Hydroxymethylfurfural	2480	ST	0.02	0.01	B	0.01	0.01	*B*	0.03	0.01	**B**	0.06	0.01	A	0.07	0.03
Ethyl esters																
Ethyl 3-ethoxypropanoate*	1320	MS[19]	0.05	0.02	A	0.04	0.03		0.11	0.07	**A**	0.01	0.01	b,B	0.01	0.01
Methyl esters																
Methyl nonanoate	1469	MS[14]	0.00	0.00	B	0.00	0.00		0.00	0.00		0.00	0.00	a,b,B	0.00	0.00
Methyl benzeneacetate***	1741	ST	0.02	0.01		0.02	0.01	*A*	0.03	0.01		0.01	0.01	a,b	0.00	0.00
Methyl hexadecanoate*	2200	MS[4]	0.02	0.01	B	0.01	0.00	*B*	0.02	0.01	**B**	0.09	0.05	a,b,AB	0.02	0.02
Methyl 9-octadecanoate*	2415	MS[14]	0.05	0.03		0.05	0.05	*A*	0.03	0.02		0.02	0.01	b	0.01	0.01
Ketones																
Diacetyl	948	ST	2.7	1.3		1.6	0.9		2.51	1.15	**A**	1.1	0.4	a	0.5	0.4
3-Heptanone*	1135	ST	0.04	0.02	a,b,A	0.2	0.3	a,*A*	0.03	0.00	b,**A**	nd	nd	B	nd	nd
2-Heptanone*	1187	ST	0.04	0.01	A	0.09	0.08	*A*	0.05	0.01	**A**	nd	nd	B	nd	nd
Acetoin	1275	ST	1.5	0.9		0.4	0.3		1.4	0.9		0.60	0.21	a	0.30	0.15
2,2,6-Trimethyl-cyclohexanone*	1292	MS[14]	0.01	0.00	B	0.00	0.00	*B*	0.02	0.02		0.01	0.00	a,b,B	0.00	0.00
2-Cyclopentenone***	1340	MS[2]	0.01	0.00	B	0.01	0.00		0.01	0.01	**B**	0.04	0.01	a,A	0.02	0.01
2-Acetylfuran	1487	ST	0.15	0.09		0.14	0.02	*A*	0.31	0.17		0.16	0.06	b	0.07	0.03
2-Methyl-2-nonen-4-one	1526	DB	0.02	0.01	A	0.01	0.00	*B*	0.03	0.03		0.02	0.01	b,A	0.01	0.00
3,4-Dihydroxy-3,4-dimethyl-2,5-hexanedione	1570	DB	0.18	0.08	A	0.12	0.03	*A*	0.14	0.02	**A**	0.05	0.01	b,B	0.02	0.01

(*continued on next page*)

Table 2 (*continued*)

Volatile compounds[1]			Samples													
			VMM									VJ				
			MMCR (n = 6)			MMRE (n = 2)			MMPX (n = 5)			JCR (n = 7)			JRE (n = 7)	
	LRI	ID[2]	RA	SD	S[3]	RA	SD	S	RA	SD	S	RA	SD	S	RA	SD
2-Hydroxy-2-cyclopenten-1-one**	1768	MS[1]	0.06	0.02	a,B	0.01	0.00	b	0.06	0.01	a	0.02	0.01	b,C	0.00	0.00
Cyclotene	1833	ST	0.03	0.01	a,AB	0.01	0.01	b,*B*	0.03	0.00	a	0.02	0.00	b,B	0.00	0.00
1-(4-Methoxyphenyl)-1-hexanone	1834	DB	0.01	0.00		0.02	0.00	*AB*	0.02	0.01		0.01	0.00	a	0.01	0.00
4-Methyl-5H-furan-2-one	1892	DB	0.03	0.01	B	0.03	0.01	*AB*	0.04	0.01		0.03	0.01	a,b,B	0.01	0.01
Benzophenone**	2455	ST	0.01	0.01		0.02	0.00	*A*	0.02	0.01	**A**	0.01	0.00	a	0.00	0.00
C_{13}-Norisoprenoids																
1,1,6-Trimethyl-1,2-dihydronaphthalene	1719	ST	0.6	0.9		0.02	0.02		1.1	1.2		0.1	0.3		0.04	0.06
β-Damascenone**	1804	ST	0.02	0.01	a,b	0.01	0.00	b	0.04	0.02	a,**B**	0.05	0.02	b	0.02	0.01
Other esters																
n-Hexyl salicylate	2198	MS[7]	0.03	0.01		0.03	0.01		0.05	0.02		0.03	0.01	a	0.01	0.01
Volatile phenols																
Guaiacol**	1849	ST	0.04	0.01	A	0.03	0.01	*A*	0.04	0.01		0.04	0.01	b,A	0.02	0.01
p-Cresol***	2083	ST	0.05	0.01	A	0.04	0.01	*A*	0.05	0.03		0.02	0.01	b,B	0.01	0.00
4-Ethylphenol**	2177	ST	0.06	0.04	B	0.06	0.00		0.09	0.05	**B**	0.16	0.07	a,b,A	0.08	0.05
Terpenes																
Camphene	1060	MS[14]	0.12	0.08	a,b	0.03	0.02	b	0.27	0.14	a	0.07	0.09		0.04	0.03
Eucalyptol**	1177	ST	0.01	0.01	B	0.01	0.00		0.02	0.02		0.01	0.00	b,B	0.00	0.00
trans-p-Mentha-2,8-dienol	1304	DB	0.01	0.00	AB	0.00	0.00		0.02	0.01		0.00	0.00	b,B	0.00	0.00
Safranal **	1627	ST	0.01	0.01		0.01	0.00		0.02	0.01		0.04	0.02		0.02	0.02
α-Terpineol **	1704	ST	0.01	0.00	B	0.01	0.00		0.02	0.01		0.01	0.00	a,b,B	0.01	0.00
Miscellaneous																
Pyrrole	1487	MS[18]	0.08	0.09		0.02	0.01		0.05	0.02	**A**	nd	nd		nd	nd
2H-Pyran, tetrahydro-2-(12-pentadecynyloxy)-	1808	DB	0.04	0.01	a,b,A	0.02	0.01	b	0.05	0.02	a	0.04	0.01	a,b,A	0.02	0.01
n.i. (m/z 85,100)	1917	MS[14]	0.06	0.01	AB	0.05	0.02	*A*	0.08	0.04		0.08	0.04	b,A	0.03	0.02
Benzothiazole	1950	MS[13]	0.02	0.00	A	0.02	0.00	*A*	0.02	0.02	**A**	nd	nd	B	nd	nd
n.i. (m/z 55,69,83)	2064	DB	0.03	0.01	B	0.02	0.00	*B*	0.03	0.01	**B**	0.04	0.01	a,b,B	0.02	0.01
n.i. (m/z 85,29,41)	2085	DB	0.06	0.02	A	0.05	0.02	*A*	0.06	0.03		0.03	0.01	b,B	0.01	0.01

Volatile compounds[1]	Samples												
	VJ			VC									TT[4]
	JPX (n = 6)			CSC (n = 5)			CSO (n = 4)			CRE (n = 8)			
	S	RA	SD	S	RA	SD	S	RA	SD	S	RA	SD	S	
Acetals														
2-Butyl-4-methyl-1,3-dioxolane		0.02	0.02		0.04	0.06		0.07	0.08		0.08	0.09		j,c
Acetates														
Methyl acetate	b	1.3	0.7	a	0.47	0.23		0.8	0.4		0.62	0.25		m,c
trans-2-Hexen-1-yl acetate	*A*	0.01	0.01	**A**	nd	nd		nd	nd		nd	nd	*B*	j
3-Oxobutan-2-yl acetate	b,*AB*	0.50	0.16	a	0.05	0.02		0.09	0.06	B	0.05	0.03	*B*	m,j,c
Acids														
2-Methylpropanoic acid**	b	0.25	0.22	a	0.23	0.13	a,b	0.52	0.25	a,A	0.21	0.17	b	j,c

(*continued on next page*)

Table 2 (*continued*)

Volatile compounds[1]	Samples													
	VJ			VC										
	JPX (n = 6)			CSC (n = 5)			CSO (*n* = 4)			CRE (*n* = 8)				TT[4]
	S	RA	SD	S	RA	SD	S	RA	SD	S	RA	SD	S	
2-Methylbutanoic acid	b	0.6	0.4	a	0.51	0.24	b	1.4	0.6	a,A	0.4	0.3	b	c
(4-Hexyl-2,5-dioxo-2,5-dihydro-3-furanyl) acetic acid*	b	0.03	0.01	a,**B**	0.07	0.04		0.07	0.04	A	0.05	0.07		j
Dodecanoic acid**		0.01	0.01		0.02	0.01		0.03	0.01	A	0.02	0.02		j,c
Alcohols														
3-Methyl-1-butanol	*B*	2.4	1.2		3.3	1.2		3.2	1.7		2.4	1.4	*AB*	m,j
3-Heptanol		0.10	0.11		0.01	0.00		0.01	0.00	B	0.01	0.01		j
2-Heptanol*	b	0.02	0.01	a,**A**	0.01	0.00		0.01	0.01	A	0.01	0.00		m
trans-2-Hexen-1-ol	*A*	0.01	0.01	**A**	nd	nd		nd	nd		nd	nd	*B*	j
1-Octen-3-ol*	b,*B*	0.04	0.02	a,**B**	nd	nd		nd	nd	B	nd	nd	*B*	m,j,c
1-Heptanol**		nd	nd		0.03	0.03		0.09	0.12	A	0.01	0.01		c
1-Nonanol**	b,*B*	0.04	0.02	a	0.02	0.01		0.03	0.02		0.01	0.01	*B*	m,c
2-Methyl-1-hexadecanol	b,*B*	0.06	0.02	a,**B**	0.02	0.01	a,b	0.04	0.02	a,B	0.01	0.01	b,*B*	m
2-Phenylethanol**		2.1	0.9		2.3	1.1		2.2	1.0		2.4	2.2		m,j
Aldehydes														
Octanal**	b,*B*	0.04	0.02	a,**B**	0.08	0.05		0.11	0.06	A	0.05	0.02	*A*	j
Benzaldehyde		0.7	0.6		1.5	1.1		2.2	1.9		1.8	1.0		m,j
trans-2-Decenal*	b	0.06	0.03	a,**A**	0.08	0.04		0.08	0.04	A	0.05	0.03		c
5-Hydroxymethylfurfural	*A*	0.08	0.03	**A**	0.02	0.00		0.03	0.01	B	0.02	0.01	*B*	j
Ethyl esters														
Ethyl 3-ethoxypropanoate*	b	0.03	0.02	a,**B**	0.00	0.00		0.02	0.04	AB	0.02	0.03		m
Methyl esters														
Methyl nonanoate	b	0.01	0.01	a	0.06	0.06		0.03	0.03	A	0.06	0.07		c
Methyl benzeneacetate***	b,*B*	0.02	0.01	a	0.01	0.00		0.03	0.02		0.01	0.01	*B*	m
Methyl hexadecanoate*	b,*B*	0.17	0.10	a,**A**	0.09	0.06		0.10	0.07	A	0.12	0.06	*A*	m
Methyl 9-octadecanoate*	b,*B*	0.05	0.02	a	0.02	0.01		0.03	0.03		0.02	0.02	*B*	m,c
Ketones														
Diacetyl	b	0.7	0.3	a,b,**B**	0.42	0.08		1.4	1.6		1.1	0.8		m
3-Heptanone*	*B*	nd	nd	**B**	nd	nd		nd	nd	B	nd	nd	*B*	m
2-Heptanone*	*B*	nd	nd	**B**	nd	nd		nd	nd	B	nd	nd	*B*	m
Acetoin	b	0.80	0.18	a	0.32	0.10		0.8	0.5		0.	0.3		m
2,2,6-Trimethyl-cyclohexanone*	b,*B*	0.01	0.01	a	0.10	0.03		0.3	0.3	A	0.17	0.10	*A*	c
2-Cyclopentenone***	b	0.05	0.02	a,**A**	0.04	0.01		0.04	0.03	A	0.03	0.01		m
2-Acetylfuran	c,*B*	0.29	0.07	a	0.08	0.02		0.15	0.13		0.13	0.05	*AB*	m,c
2-Methyl-2-nonen-4-one	b,*A*	0.04	0.02	a	nd	nd		nd	nd	B	nd	nd	*C*	c
3,4-Dihydroxy-3,4-dimethyl-2,5-hexanedione	b,*B*	0.08	0.03	a,**B**	0.07	0.03		0.15	0.08	A	0.09	0.06	*AB*	m,j,c
2-Hydroxy-2-cyclopenten-1-one**	b	0.05	0.03	a	0.05	0.01	b	0.10	0.04	a,A	0.03	0.01	b	j
Cyclotene	c,*B*	0.04	0.02	a	0.04	0.01	a,b	0.07	0.05	a,A	0.03	0.01	b,*A*	j,c
1-(4-Methoxyphenyl)-1-hexanone	b,*B*	0.02	0.01	a	0.03	0.01		0.06	0.06		0.03	0.02	*A*	c
4-Methyl-5H-furan-2-one	b,*B*	0.04	0.01	a	0.05	0.01		0.08	0.06	A	0.04	0.02	*A*	j,c
Benzophenone**	b,*B*	0.00	0.00	b,**B**	0.02	0.01		0.02	0.01		0.01	0.01	*AB*	j
C_{13}-Norisoprenoids														
1,1,6-Trimethyl-1,2-dihydronaphthalene		0.3	0.4		0.4	0.5		0.4	0.2		0.16	0.23		m,j

(*continued on next page*)

Table 2 (*continued*)

Volatile compounds[1]	Samples													
	VJ			VC										
	JPX (n = 6)			CSC (n = 5)			CSO (*n* = 4)			CRE (*n* = 8)				TT[4]
	S	RA	SD	S	RA	SD	S	RA	SD	S	RA	SD	S	
β-Damascenone**	c	0.09	0.03	a,**A**	0.02	0.00		0.04	0.04		0.02	0.01		j
Other esters														
n-Hexyl salicylate	b	0.04	0.01	a	0.05	0.01		0.06	0.04		0.03	0.01		j,c
Volatile phenols														
Guaiacol**	c,*AB*	0.06	0.02	a	0.01	0.00		0.01	0.01	B	0.01	0.01	*B*	c
p-Cresol***	c,*B*	0.04	0.01	a	0.02	0.00		0.02	0.02	B	0.01	0.01	*B*	m
4-Ethylphenol**	b	0.22	0.09	a,**A**	0.06	0.05		0.07	0.05	B	0.11	0.13		m,j
Terpenes														
Camphene		0.14	0.12		0.05	0.02		0.05	0.03		0.03	0.03		m
Eucalyptol**	b	0.01	0.00	a	0.05	0.03		0.05	0.04	A	0.04	0.04		c
trans-p-Mentha-2,8-dienol	b	0.01	0.00	a	0.01	0.01		0.01	0.01	A	0.01	0.01		j
Safranal **		0.05	0.02		0.05	0.01		0.04	0.04		0.04	0.03		m
α-Terpineol **	b	0.01	0.00	a	0.03	0.01		0.04	0.03	A	0.03	0.02		c
Miscellaneous														
Pyrrole		nd	nd	**B**	0.16	0.15		0.08	0.08		0.4	0.6		j,c
2H-Pyran, tetrahydro-2-(12-pentadecynyloxy)-	b	0.05	0.02	a	0.02	0.01		0.02	0.01	B	0.01	0.01		c
n.i. (m/z 85,100)	b,*AB*	0.13	0.05	a	0.02	0.01		0.02	0.03	B	0.01	0.01	*B*	c
Benzothiazole	B	nd	nd	**B**	nd	nd		nd	nd	B	nd	nd	*B*	m
n.i. (m/z 55,69,83)	b,*B*	0.06	0.02	a,**A**	0.08	0.02		0.12	0.10	A	0.06	0.02	*A*	c
n.i. (m/z 85,29,41)	b,*B*	0.05	0.02	a	0.01	0.01		0.02	0.01	B	0.01	0.00	*B*	m,j,c

ID (identification): reliability of identification: ST, mass spectrum and LRI agreed with standards (own or literature); MS, mass spectrum agreed with mass spectral data base and LRI agreed with the literature data; DB, mass spectrum agreed with mass spectral data base.

RA: Mean relative areas. SD: Standard deviation. S: Significant differences.

S[3]: Different lowercase letters in different columns indicate significant differences according to Tukey's test ($p < .05$) between categories within each PDO. Different capital letters in different columns indicate significant differences according to Tukey's test ($p < .05$) between similar categories of different PDOs: A, B, C = Significant difference between JCR, MCR and CSO; *A, B, C* = Significant difference between JRE, MRE and CRE; **A, B** = Significant difference between JPX and MPX.

TT[4]: Tukey's test ($p < .05$) among PDOs. Letters indicate: m,j = significant difference between VJ and VM; j,c = significant difference between VJ and VC; m,c = significant difference between VC and VM; m,j,c = significant difference between all PDOs; j = significant difference between VJ and the others; c = significant difference between VC and the others; m = significant difference between VM and the others.

nd: Peak not detected; n.i: non identified peak.

[1] : *detected in product derived from grape and for the first time in vinegars; **detected for the first time in "Vinagre de Condado de Huelva" and "Vinagre de Montilla-Moriles" PDOs; ***detected for the first time in "Vinagre de Jerez" PDO.

[2] Number in superscript in this column correspond to the reference were the compounds has also been identified :1: Antoniotti, Alezra, Fernandez & Dunach, 2004; 2: Chevance & Farmer, 1999; 4: Hanai & Hong, 1989; 7: Lukic, Radeka, Grozaj, Staver, & Persuric, 2016; 8: Marrufo-Curtido et al., 2012; 13: Pozo-Bayon, Ruiz-Rodriguez, Pernin & Cayot, 2007; 14: Pubchem database 2005; 16: Rychlik, Schieberle & Grosch, 1998; 18: Shimoda, Shigematsu, Shiratsuchi & Osajima, 1995; 19: Ubeda, et al., 2016. (Complete references in the reference list).

in our case, samples of different categories were obtained from different producers and wineries.

3.2. Characterisation, comparison and classification of the Spanish PDO wine vinegars

Regarding the ANOVA performed considering the PDOs taken as a whole, 57 of the 160 total compounds showed significant differences between the three PDOs (Table 2), indicated with a letter or some letters (i.e. j, m, c) in the last column of the table. Forty-one compounds of these 57 showed significant differences between one PDO from the rest and even between the three PDOs and are shown in Table 2. Moreover, 4 of these compounds showed significant differences between the three PDOs (Table 2). Moreover, among the 57 compounds with significant difference between PDOs, 10 were detected in only one or two of the three PDOs under study (e.g. 2- and 3-heptanone were present in VMM only) and in general, taking into account the total RA for the different groups, ketones, C_{13}-norisoprenoids and volatile phenols were the three families of compounds that showed significant between-PDO differences.

On the one hand, in order to evidence the overall impact of the volatile composition on each PDO, a heatmap was performed considering the 41 significant volatile compounds above mentioned (Fig. 2).

Hence, the results of the heatmap showed that the compounds which were identified as the most significant for differentiating VC PDO from the other two PDOs were: 1-heptanol, methyl nonanoate, 2-methylbutanoic acid, 2,2,6-trimethyl-cyclohexanone, *trans*-2-decenal, eucalyptol, α-terpineol, and the compound with ions 55,69,83 (Fig. 2). Among them, 2-methylbutanoic acid, *trans*-2-decenal and 2,2,6-trimethyl-cyclohexanone achieved significantly higher RA values in this VC PDO with relation to the other two PDOs (Table 2). These compounds were detected for the first time in this PDO. Moreover, methyl nonanoate, eucalyptol and α-terpineol appeared to be more closely-related to the VC PDO due to they presented higher RA in VC PDO than in the other two PDOs. Regarding 1-heptanol, it was detected only in the VC PDO and also for the first time in vinegars. This compound is a yeast metabolite produced during alcoholic fermentation (Cacho, Campillo, Viñas, & Hernández-Córdoba, 2014).

Regarding the VMM PDO, the compounds identified as being the most significant for this PDO according to heatmaps results were: diacetyl and acetoin, ethyl 3-ethoxypropanoate, 2- and 3-heptanone, 2-methyl-1-hexadecanol, 1-octen-3-ol, *p*-cresol, benzothiazole and camphene, followed by 3-oxobutan-2-yl acetate (also known as acetoin acetate) and 3,4-dihydroxy-3,4-dimethyl-2,5-hexanedione (TI) (Fig. 2). Within them, diacetyl and acetoin stood out due to their high RAs in this PDO, mainly in the less aged category (MMCR) and the sweet category (MMPX) (Table 2). Acetoin has been described as the odorant showing the highest content in musts from *Pedro Ximénez* sun-dried grapes (Ruiz, Zea, Moyano, & Medina, 2010). Its high RA values in MMPX category could, therefore, be explained by the fact that this sweet vinegar is produced by adding this type of must, according to the PDO's regulations (Council Regulation (EC) N° 510/2006). Moreover, 1-octen-3-ol and 2-methyl-1-hexadecanol, ethyl 3-ethoxypropanoate and camphene also appeared to be more related to VMM PDO than to the other two PDOs, due to their high RA values (Table 2). Regarding 1-octen-3-ol, it has been reported in PX grape must from the PDO Montilla-Moriles (Morales, Fierro-Risco, Ríos-Reina, Ubeda, & Paneque, 2019). On the other hand, although 3-oxobutan-2-yl acetate and 3,4-dihydroxy-3,4-dimethyl-2,5-hexanedione showed significant differences among the three PDOs, they achieved the highest RA values in VMM PDO, being also identified for the first time in vinegars. The high presence of acetoin and the trend observed for acetates in this PDO could explain the high RA values of 3-oxobutan-2-yl acetate in these vinegars. In addition to these characteristic compounds of VMM PDO, it could be highlighted that three compounds (3-heptanone, 2-heptanone and benzothiazole) were only determined in VMM samples. These compounds have been identified as volatile constituents from grapes (Schreier, Drawert, & Junker, 1976).

Lastly, the relevant volatile compounds identified by the heatmap for distinguishing the VJ PDO samples were: β-damascenone, 5-hydroxymethylfurfural, 3-heptanol, *trans*-2-hexen-1-ol and *trans*-2-hexen-1-yl acetate (Fig. 2). 5-hydroxymethylfurfural is worth noting (Table 2). This is a compound that is a result of both the wooden barrel ageing process (Tesfaye, Morales, García-Parrilla, & Troncoso, 2002), and a product from Maillard reactions (Ortega-Heras & González-Sanjosé, 2009). For this reason, its higher RA values in VJ PDO could be due to a higher ageing of the samples or in the case of JPX samples, due to the wines used for the production of this category were aged and pacified, being different in MPX as was discussed above. Moreover, the compounds detected only in this PDO were *trans*-2-hexen-1-ol and its ester, *trans*-2-hexen-1-yl acetate (TI), this last being previously determined in JCR samples by Marrufo-Curtido et al. (2012). However, the alcohol *trans*-2-hexen-1-ol had never been determined in JCR and JRE categories, and it could be derived from the specific variety of grape used for these vinegars. In addition, 3-heptanol appeared to be more closely-related to the VJ PDO, specially due to the high RA values in JPX.

In addition, according to the results showed in Table 2, another important aspect to highlight that could help in the differentiation of the PDOs was the absence of certain compounds in one of the PDOs. Thus, 2-methyl-2-nonen-4-one (TI) and 1-octen-3-ol were not detected in VC PDO, 2-heptanol was not present in the VMM PDO, probably due to the aforementioned high presence of the ketones derived from this compound in this PDO, and finally, pyrrole was the volatile compound not detected in the VJ PDO and detected in the other two PDOs.

On the other hand, PLS-DA models were carried out for studying the ability of the volatile composition to discriminate and classify the wine vinegars according to the three Spanish PDOs. Thus, in the first stage, a PLS-DA classification model based on a 4 latent-variables (LVs) was built on the data comprising the 160 compounds extracted by MCR. Then, in order to find the best and simplest classification model with fewer variables, a PLS-DA model based only on 3 latent variables, chosen according to minimum cross-validation classification errors, was built using the 41 volatile compounds previously discussed (i.e. those that showed significant differences between one PDO from the rest and even between the three PDOs). Results of these two classification models are shown in Table 3. Moreover, the score plots of the two PLS-DA models are shown in Fig. 3. The dataset was split into a training and a test set of 33 and 17 samples, respectively. The models were built and optimised for the training samples by selecting the best pre-treatment and optimum number of latent variables (LVs) to be retained (leading to the lowest classification error in venetian blind cross-validation) and then validated by analysing the test set and evaluating predictive accuracy. The classification results obtained (Table 3) showed promising classification results for both models. This reaffirms the utility of the proposed methodology for classifying and authenticating wine vinegar PDOs. However, in order to compare and select the best model, Table 3 and Fig. 3 illustrate that the second model, with only the 41 selected compounds, showed an improvement in the classification rates with respect to the model obtained for the total data. Thus, 100% of PDO samples were correctly classified in the '41-selected volatile model', whereas in the other model the results showed some classification errors. Moreover, the total variance explained by the two models with the minimum number of latent variables is a parameter that is also useful for comparing the said models. Thus, a higher percentage of total variance explained by fewer LVs (71.45%) was obtained using the second model.

Furthermore, to identify the variables most effective in classifying the PDO samples, the values of the variable importance in projection (VIP) index were calculated and the results are showed in Table 4. VIP is scaled in such a way that all of the predictors having a VIP > 1 are considered to be relevant (Mehmood, Liland, Snipen, & Sæbø, 2012). In

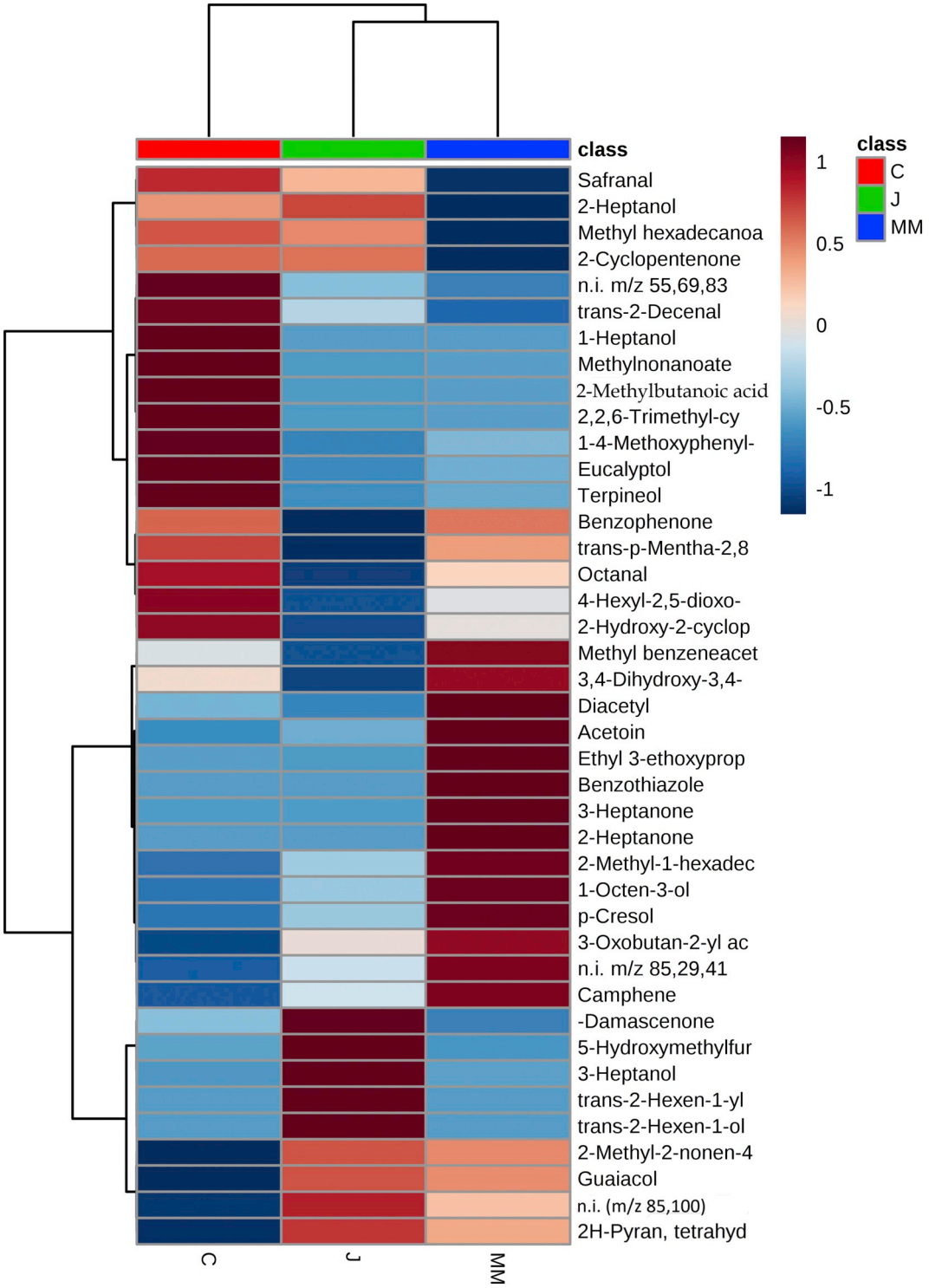

Fig. 2. Hierarchical clustering and heatmap for each PDO performed by Pearson correlation coefficient. Note: J: *"Vinagre de Jerez"* PDO, C: *"Vinagre de Condado de Huelva"* PDO, MM: *"Vinagre de Montilla-Moriles"* PDO.

particular, the VIP scores obtained for the '41-selected volatile model' confirmed that the compounds highlighted in the heatmap and previously discussed, were again the most relevant for the classification of the three PDO wine vinegars.

All of the stated compounds that have been related to each PDO, according to the results obtained through all of the statistical analysis performed, could be responsible for a specific and different aromatic profile for each PDO, and hence, they could be considered as possible markers of authenticity of these PDO wine vinegars. In addition, these differences in volatile composition between the three PDOs could lead to differences in the overall aroma of these vinegars. In consequence, further studies using information on odour thresholds of these compounds and their study by GC-olfactometry and sensory analysis would be needed to confirm this hypothesis.

Table 3
Sensitivity, specificity and classification errors (%) obtained for (a) PLS-DA classification models to differentiate PDOs by the total of MCR compounds; (b) PLS-DA classification models to differentiate PDOs by the inclusion of the selected compounds. The acronyms for the different vinegar categories are defined in Table 1.

	a) PLS-DA "160-volaitle compounds" model			b) PLS-DA "41-selected volaitle compounds" model		
N° of LVs	4			3		
Cross validation	venetian blinds w/ 5 splits					
% total variance	60.85			71.45		
PDO	VC	VJ	VMM	VC	VJ	VMM
Sensitivity (Cal)	100	100	100	100	100	100
Specificity (Cal)	100	100	100	100	100	100
Sensitivity (CV)	100	100	100	100	100	100
Specificity (CV)	100	90.0	100	100	100	100
Sensitivity (Pred)	100	100	100	100	100	100
Specificity (Pred)	100	100	100	100	100	100
Correct classified (Cal)	100	100	100	100	100	100
Correct classified (CV)	100	95.0	87.5	100	100	100
Correct classified (Pred)	100	100	100	100	100	100

4. Conclusions

A detailed and comprehensive characterisation, differentiation of the volatile profiles and classification of the three Spanish PDO wine vinegars, *Vinagre de Jerez*, *Vinagre de Condado de Huelva* and *Vinagre de Montilla-Moriles,* considering their most commercialized categories, have been studied for the first time by using the same methodology, Headspace Stir Bar Sorptive Extraction (HSSE) in conjunction with Gas Chromatography-Mass Spectrometry (GC–MS) and chemometrics.

Although the volatile composition of the VJ PDO was widely studied in comparison with the VMM and VC PDOs, 7 new compounds have been determined for the first time in these PDO wine vinegar, 4 being identified by standards. In addition, 28 compounds have also been reported for the first time in VC and VMM PDOs.

Moreover, the three wine vinegar PDOs and their categories presented significant differences in their volatile profiles, in spite of showing similar total number of compounds. Thus, acetates were the majority group of compounds in all of the categories, showing higher values in the sweet categories (Pedro Ximénez), whereas ketones, C_{13}-norisoprenoids and volatile phenols, in spite of being minor volatile compounds in grape derived products, were the three families of compounds that showed significant between-PDO differences. Regarding the different categories studied in general, acids and ketones showed higher RA values in *Crianza* samples, whereas several ethyl esters showed higher RA values in *Reserva* samples. Moreover, compounds grouped as acetates, volatile phenols and terpenes were more closely-related to *Pedro Ximénez* samples according to their high RA values. Within each PDO, there were also significant differences between their volatile profiles.

The satisfactory results obtained by partial least squares-discriminant analysis (PLS-DA) reaffirm the utility of the volatile profile for differentiating, classifying and authenticating wine vinegar PDOs by only the need of a few compounds, that could be considered as markers. Thus, according to the heatmap and VIPs, 1-heptanol, methyl nonanoate, 2-methylbutanoic acid, 2,2,6-trimethyl-cyclohexanone, *trans*-2-decenal, eucalyptol and α-terpineol, were identified as the most significant compounds for the differentiation of the VC PDO; diacetyl, acetoin, ethyl 3-ethoxypropanoate, 2- and 3-heptanone, 2-methyl-1-hexadecanol, 1-octen-3-ol, *p*-Cresol and camphene for the VMM PDO and finally, β-damascenone, 5-hydroxymethylfurfural, 3-heptanol, *trans*-2-hexen-1-ol and *trans*-2-hexen-1-yl acetate for the VJ PDO. These possible authenticity markers could contribute to the specific aromatic profile of each PDO. For this reason, further studies are being developed to determine the aromatic impact of these markers in the overall aroma of the PDO wine vinegars.

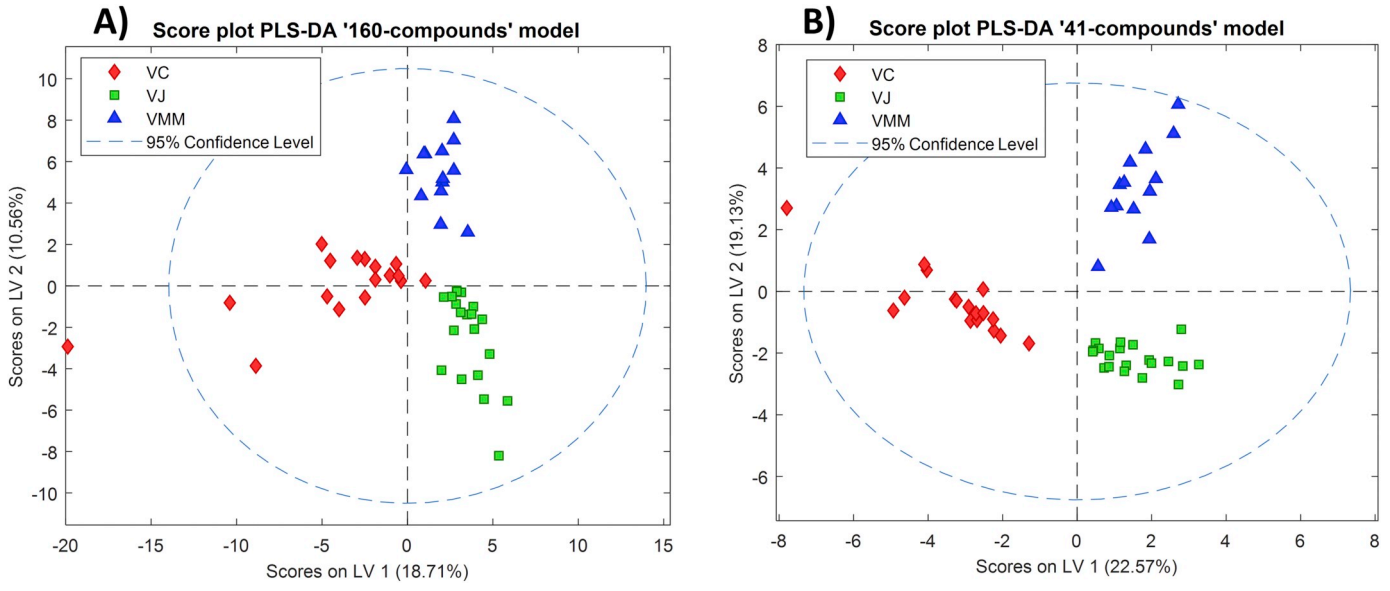

Fig. 3. Score plots of the two PLS-DA classification models: one built on the data composed by the 160 compounds extracted by MCR (A) and the other built using the 41 selected volatile compounds (B).

Table 4
Score values of the variable importance in projection (VIP) index for each PDO obtained by the "41-selected-compounds" PLS-DA classification model. Note: Values in bold are considered relevant variables for the classification of PDO samples.

Variable	VIP Scores for VC	VIP Scores for VJ	VIP Scores for VM
3-Oxobutan-2-yl acetate	**1.64**	1.11	0.99
2-Methylbutanoic acid	0.96	0.37	0.17
n.i. (*m/z* 55,69,83)	**1.13**	0.59	0.35
(4-Hexyl-2,5-dioxo-2,5-dihydro-3-furanyl) acetic acid	**1.14**	0.74	0.64
3-Heptanol	0.48	0.80	0.92
2-Heptanol	0.12	0.24	0.22
1-Octen-3-ol	0.04	1.99	**2.72**
2-Methyl-1-hexadecanol	1.22	1.50	1.67
Octanal	**1.28**	0.93	0.85
trans-2-Decenal	**1.59**	0.98	0.77
Safranal	0.48	0.80	0.86
5-Hydroxymethylfurfural	1.66	2.75	**2.98**
Ethyl 3-ethoxypropanoate	0.23	0.95	**1.22**
Methyl nonanoate	**1.70**	0.62	0.28
Methyl benzeneacetate	0.01	0.76	**1.01**
Methyl hexadecanoate	0.33	0.91	1.10
Diacetyl	0.11	1.48	**2.00**
Acetoin	0.30	1.07	**1.37**
2-Cyclopentenone	0.15	1.64	**2.00**
2-Methyl-2-nonen-4-one	0.07	0.15	0.10
2-Hydroxy-2-cyclopenten-1-one	0.59	0.58	0.59
Benzophenone	0.79	1.13	**1.29**
3,4-Dihydroxy-3,4-dimethyl-2,5-hexanedione	0.06	1.53	**2.08**
n.i. (m/z 85,29,41)	1.64	1.30	1.25
β-Damascenone	0.54	1.56	1.71
Guaiacol	**2.57**	1.09	0.57
p-Cresol	0.94	1.15	1.27
Eucalyptol	**2.15**	0.79	0.37
Camphene	0.57	0.65	0.70
trans-p-Mentha-2,8-dienol	0.41	0.57	0.65
α-Terpineol	**2.18**	0.82	0.39
n.i. (m/z 85,100)	**2.01**	1.09	0.74
2,2,6-Trimethyl-cyclohexanone	**2.46**	0.91	0.42
2H-Pyran, tetrahydro-2-(12-pentadecynyloxy)-	**2.05**	0.88	0.45
1-(4-Methoxyphenyl)-1-hexanone	**1.41**	0.57	0.27

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2019.04.071.

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Table I. Supplementary Material. Mean, standard deviations and significant differences according to Tukey's test (p<0.05) for each category of the relative areas (RA) of the 160 total detected and identified compounds.

				Samples																															
				VMM											VJ									VC												
	Volatile compounds[1]			MMCR (n=6)			MMRE (n=2)			MMPX (n=5)			JCR (n=7)			JRE (n=7)			JPX (n=6)			CSC (n=5)			CSO (n=4)			CRE (n=8)			TT[4]
		LRI	ID[2]	RA	SD	S[3]	RA	SD	S	RA	SD	S	RA	SD	S	RA	SD	S	RA	SD	S	RA	SD	S	RA	SD	S	RA	SD	S	
	Acetals																																		
1	2,4,5-Trimethyl-1,3-dioxolane	924	MS[9]	0.9	0.6		1.5	1.0		0.9	0.6		0.5	0.5		0.6	0.3		0.8	0.7		0.31	0.25		2	3		1.3	1.4		
2	4,5-Dimethyl-2-pentadecyl-1,3-dioxolane	1109	DB	0.4	0.5		0.10	0.01		0.4	0.3		0.10	0.05		0.14	0.15		0.3	0.3		0.11	0.13		0.4	0.5		0.3	0.4		
3	1,1-Diethoxyisobutane*	1225	MS[20]	0.01	0.01		0.00	0.00		0.01	0.01		0.01	0.01	b	0.01	0.01	a,b	0.05	0.04	a	0.00	0.00		0.04	0.06		0.04	0.03		
4	2-Butyl-4-methyl-1,3-dioxolane	1260	DB	0.04	0.04		0.01	0.01		0.05	0.03		0.01	0.01		0.02	0.04		0.02	0.02		0.04	0.06		0.07	0.08		0.08	0.09		j,c
5	2-Methoxymethyl-2,4,5-trimethyl-1,3-dioxolane	1435	DB	0.12	0.08		0.10	0.09		0.14	0.08		0.04	0.03	a,b	0.04	0.02	b	0.12	0.09	a	0.03	0.01		0.10	0.11		0.08	0.06		
	Total of acetals			1.47	1.17		1.69	1.14		1.5	1.0		0.7	0.6		0.8	0.5		1.30	1.14		0.5	0.5		2.3	3.6		1.77	2.00		
	Acetates																																		
6	Methyl acetate	871	ST	1.2	0.7		0.59	0.21		1.2	0.4		1.0	0.4	a,b	0.5	0.4	b	1.3	0.7	a	0.47	0.23		0.8	0.4		0.62	0.25		m,c
7	Ethyl Acetate	903	ST	29.0	11.5		34.8	13.7		34.7	9.0	**B**	28	23	b	20	14	b	65	27	a,**A**	27.19	14.33		34	45		30.71	20.00		
8	Propyl acetate	945	ST	0.32	0.17		0.4	0.4		0.25	0.13		0.3	0.4		0.6	0.7		1.0	0.9		0.30	0.21		1.0	1.9		0.9	0.8		
9	2-Methylpropyl acetate	973	ST	4	3		2.8	1.1		3.8	1.3		2.3	1.2		2.1	1.9		4	3		3.1	1.3		5	6		3.3	2.1		
10	Butyl acetate	1027	MS[15]	0.25	0.14		0.5	0.6		0.38	0.22		0.09	0.10	b	0.14	0.16	a,b	0.33	0.20	a	0.09	0.07		0.4	0.7		0.26	0.24		
11	3-Methylbutyl acetate	1085	ST	17	13		10	4		11.3	1.6		7	5		6	5		13	12		8	4		16	22		10	10		
12	2-Ethylbutyl acetate	1091	ST	0.02	0.01		0.01	0.00		0.01	0.00		0.01	0.01		0.02	0.04		0.03	0.02		0.02	0.01		0.03	0.03		0.01	0.01		
13	Hexyl acetate	1245	ST	0.05	0.03		0.05	0.06		0.07	0.03		0.11	0.23		0.03	0.06		0.13	0.16		0.07	0.11		0.3	0.5		0.3	0.4		
14	4-Hexen-1-yl acetate	1289	DB	0.02	0.02		0.01	0.00		0.02	0.01		0.02	0.02		0.02	0.01		0.04	0.03		0.02	0.02		0.02	0.04		0.02	0.03		

15	*trans*-2-Hexen-1-yl acetate	1307	MS[8]	nd	nd		nd	nd	*B*	nd	nd	**B**	0.02	0.03		0.00	0.00	*A*	0.01	0.01	**A**	nd	nd		nd	nd		nd	nd	*B*		j
16	But-3-ene-1,2-diyl diacetate	1354	DB	0.13	0.09		0.09	0.06		0.12	0.13		0.06	0.05		0.05	0.03		0.10	0.08		0.01	0.00		0.15	0.25		0.12	0.10								
17	2-Ethylhexyl acetate*	1361	MS[6]	0.07	0.03		0.17	0.17	*A*	0.07	0.05		0.03	0.03	b	0.02	0.01	b,*B*	0.08	0.06	a	0.05	0.03		0.09	0.09		0.04	0.03	*B*							
18	3-Oxobutan-2-yl acetate	1362	ST	0.7	0.4	A	0.34	0.23	*A*	0.7	0.4		0.28	0.08	b,AB	0.18	0.09	b,*A B*	0.50	0.16	a	0.05	0.02		0.09	0.06	B	0.05	0.03	*B*		m,j, c					
19	Benzyl acetate	1709	ST	0.17	0.09		0.17	0.11		0.4	0.4		0.17	0.08	b	0.10	0.05	b	0.33	0.12	a	0.09	0.04		0.4	0.7		0.30	0.23								
20	2-Phenylethyl acetate	1796	ST	4.6	2.5		5.0	1.9		3.7	0.6	**B**	5.6	2.8	a,b	3.5	1.9	b	8.6	4.0	a,**A**	5.7	3.5		7.02	4.95		5.89	4.79								
	Total of acetates			58	32		55	22		57	14		44	33		33	25		96	48		45	24		66	83		53	39								
	Acids																																				
21	Benzoic acid***	1150	ST	0.03	0.02	a	0.00	0.00	b	0.00	0.00	b,**B**	0.05	0.04		0.02	0.01		0.04	0.04	**A**	0.02	0.02		0.05	0.04		0.05	0.06								
22	Acetic acid	1437	ST	6.3	4.0		0.35	0.20		1.6	1.2		1.7	1.6		0.7	0.4		5.1	6.3		2.03	1.3	b	7	5	a	3.0	2.6	a,b							
23	Propanoic acid	1527	MS[10]	0.06	0.04	AB	0.00	0.00		0.02	0.02		0.02	0.01	B	0.01	0.01		0.05	0.05		0.02	0.01		0.07	0.04	A	0.04	0.04								
24	2-Methylpropanoic acid**	1557	ST	0.31	0.20	AB	0.02	0.02		0.22	0.12		0.09	0.07	a,b,B	0.03	0.03	b	0.25	0.22	a	0.23	0.13	a,b	0.52	0.25	a,A	0.21	0.17	b		j,c					
25	Butanoic acid	1619	ST	0.07	0.05		0.01	0.00		0.05	0.04		0.04	0.04		0.01	0.01		0.09	0.08		0.03	0.02		0.12	0.09		0.3	0.5								
26	3-Methylbutanoic acid**	1661	MS[19]	1.9	1.3	AB	0.07	0.05		1.8	0.7		0.7	0.7	a,b,B	0.20	0.13	b	1.9	1.4	a	1.9	1.1	a,b	2.9	0.7	a,A	1.2	1.1	b							
27	2-Methylbutanoic acid	1662	ST	0.43	0.19	a,B	0.03	0.02	b	0.35	0.17	a,b	0.18	0.15	b,B	0.06	0.04	b	0.6	0.4	a	0.51	0.24	b	1.4	0.6	a,A	0.4	0.3	b	c						
28	Pentanoic acid	1736	ST	0.02	0.01	A	0.00	0.00		0.02	0.01		0.00	0.00	B	0.00	0.00		0.02	0.02		0.01	0.01		0.03	0.01	A	0.02	0.02								
29	Hexanoic acid	1841	ST	0.11	0.07	AB	0.00	0.00		0.11	0.08		0.04	0.03	a,b,B	0.02	0.01	b	0.12	0.12	a	0.08	0.05		0.17	0.08	A	0.07	0.07								
30	2-Ethylhexanoic acid**	1946	ST	0.03	0.02	b,AB	0.00	0.00	b	0.11	0.05	a	0.01	0.01	b,B	0.01	0.01	b	0.07	0.03	a	0.04	0.03		0.05	0.04	A	0.02	0.02								
31	Heptanoic acid**	1948	ST	0.02	0.01	a,b	0.00	0.00	b	0.03	0.02	a	0.01	0.01		0.00	0.00		0.03	0.03		0.01	0.01		0.05	0.07		0.01	0.01								
32	Octanoic acid	2057	ST	0.11	0.05	a,AB	0.00	0.00	b	0.06	0.03	a,b	0.05	0.04	B	0.10	0.17		0.09	0.08		0.07	0.04		0.15	0.05	A	0.10	0.09								
33	(4-Hexyl-2,5-dioxo-2,5-dihydro-3-furanyl) acetic acid*	2098	DB	0.04	0.02	a,b,A	0.00	0.00	b	0.07	0.04	a,**A**	0.01	0.00	b,B	0.01	0.00	b	0.03	0.01	a,**B**	0.07	0.04		0.07	0.04	A	0.05	0.07			j					
34	Nonanoic acid	2165	ST	0.04	0.02	a	0.00	0.00	b	0.02	0.01	a,b	0.02	0.01		0.01	0.01		0.04	0.04		0.02	0.01		0.03	0.02		0.02	0.02								
35	Decanoic acid	2275	ST	0.10	0.05	a	0.01	0.01	b	0.05	0.02	a,b	0.04	0.04		0.2	0.3		0.06	0.06		0.05	0.03		0.09	0.04		0.15	0.18								

36	Dodecanoic acid**	2456	ST	0.02	0.01	a,A	0.01	0.00	b	0.02	0.00	a,b	0.01	0.00	B	0.01	0.01		0.01	0.01		0.02	0.01		0.03	0.01	A	0.02	0.02		j,c
	Total of acids			10	6		0.5	0.3		4.5	2.5		3	3		1.4	1.2		8.5	8.9		5	3		13	7		6	5		
	Alcohols																														
37	Ethanol	932	ST	1.8	0.9		4	4		3.5	2.2		2	3		1.6	1.5		5	4		2.7	1.5		3	4		4	3		
38	3-Methyl-1-butanol	1197	ST	5	4		4.1	2.3	*A*	3.7	1.2		2.5	1.2		1.4	1.1	*B*	2.4	1.2		3.3	1.2		3.2	1.7		2.4	1.4	*AB*	m,j
39	2-Methyl-1-butanol	1199	ST	3.7	1.8		2.9	1.0		3.5	0.7		3.0	2.4		2.4	2.3		4	4		1.9	1.1		2.2	2.6		2.5	2.3		
40	3-Heptanol	1223	MS[14]	0.01	0.00	B	0.01	0.00		0.01	0.00		0.04	0.03	A	0.02	0.01		0.10	0.11		0.01	0.00		0.01	0.00	B	0.01	0.01		j
41	2-Heptanol*	1310	ST	nd	nd	B	nd	nd		nd	nd	**B**	0.01	0.01	a,b,A	0.01	0.01	b	0.02	0.01	a,**A**	0.01	0.00		0.01	0.01	A	0.01	0.00		m
42	1-Hexanol	1344	ST	0.03	0.01		0.04	0.04		0.04	0.02		0.10	0.20		0.03	0.04		0.09	0.09		0.04	0.04		0.07	0.10		0.10	0.15		
43	*trans*-2-Hexen-1-ol	1395	ST	nd	nd		nd	nd	*B*	nd	nd	**B**	0.02	0.02		0.01	0.00	*A*	0.01	0.01	**A**	nd	nd		nd	nd		nd	nd	*B*	j
44	1-Octen-3-ol*	1439	ST	0.09	0.02	A	0.08	0.04	*A*	0.11	0.04	**A**	0.02	0.01	b,B	0.01	0.00	b,*B*	0.04	0.02	a,**B**	nd	nd		nd	nd	B	nd	nd	*B*	m,j,c
45	1-Heptanol**	1448	ST	nd	nd	B	nd	nd		nd	nd		nd	nd	B	nd	nd		nd	nd		0.03	0.03		0.09	0.12	A	0.01	0.01		c
46	2-Ethyl-1-hexanol	1489	ST	0.19	0.11	AB	0.5	0.5	*A*	0.21	0.17		0.15	0.09	a,b,B	0.07	0.03	b,*B*	0.23	0.13	a	0.29	0.12		0.36	0.20	A	0.20	0.09	*B*	
47	1-(2-Methoxypropoxy)-2-propanol	1526	MS[14]	0.01	0.00	b	0.03	0.00	a,b, *A*	0.05	0.03	a	0.01	0.01	a,b	0.01	0.01	b,*B*	0.03	0.01	a	0.02	0.01		0.04	0.04		0.02	0.01	*AB*	
48	Furfuryl alcohol	1653	MS[11]	0.14	0.04		0.12	0.04		0.13	0.06		0.14	0.04	a,b	0.07	0.04	b	0.19	0.10	a	0.13	0.04		0.18	0.13		0.10	0.03		
49	1-Nonanol**	1655	ST	0.03	0.01		0.03	0.03	*A*	0.03	0.01		0.03	0.01	b	0.01	0.01	b,*B*	0.04	0.02	a	0.02	0.01		0.03	0.02		0.01	0.01	*B*	m,c
50	2-Methyl-1-hexadecanol	1749	DB	0.09	0.02	A	0.07	0.01	*A*	0.12	0.05	**A**	0.05	0.02	a,B	0.02	0.01	b,*B*	0.06	0.02	a,**B**	0.02	0.01	a,b	0.04	0.02	a,B	0.01	0.01	b,*B*	m
51	2-Methyl-1-decanol	1789	DB	0.06	0.02	a,b	0.02	0.00	b	0.08	0.04	a	0.08	0.03	a,b	0.03	0.03	b	0.12	0.05	a	0.06	0.03	a	0.07	0.03	a	0.02	0.01	b	
52	1-Undecanol	1856	DB	0.25	0.09	a,b	0.06	0.03	b	0.33	0.15	a	0.22	0.09		0.2	0.3		0.19	0.10		0.26	0.11	a	0.22	0.09	a,b	0.10	0.06	b	
53	Benzyl alcohol**	1873	ST	0.07	0.03		0.08	0.04		0.18	0.18		0.09	0.03	a	0.04	0.02	b	0.10	0.03	a	0.05	0.01		0.11	0.15		0.11	0.07		

54	2-Phenylethanol**	1909	ST	2.8	1.2		3.4	1.1		2.5	0.7		2.0	0.8		1.1	0.7		2.1	0.9		2.3	1.1		2.2	1.0		2.4	2.2		m,j
55	1-Dodecanol*	1961	ST	0.19	0.05		0.16	0.06	*A*	0.23	0.11		0.17	0.05	a	0.05	0.03	b,*B*	0.22	0.10	a	0.26	0.12		0.24	0.18		0.14	0.04	*A*	
56	1-Hexadecanol*	2370	MS[5]	0.13	0.04		0.12	0.05	*A*	0.14	0.06		0.14	0.04	b	0.05	0.03	c,*B*	0.23	0.10	a	0.14	0.05		0.13	0.09		0.08	0.03	*AB*	
	Total of alcohols			15	9		16	9		15	6		11	8		7	6		15	11		12	5		12	10		13	9		
	Aldehydes																																			
57	Octanal**	1262	ST	0.06	0.02	B	0.04	0.01	*A*	0.07	0.02	**A**	0.02	0.01	b,B	0.01	0.00	b,*B*	0.04	0.02	a,**B**	0.08	0.05		0.11	0.06	A	0.05	0.02	*A*	j
58	Nonanal**	1370	ST	0.05	0.02	B	0.06	0.02		0.08	0.05	**B**	0.12	0.03	b,AB	0.04	0.03	b	0.21	0.11	a,**A**	0.13	0.08		0.15	0.09	A	0.07	0.03		
59	2-Furfuraldehyde	1437	ST	1.0	0.4		0.69	0.19		1.7	0.9		1	1	a,b	0.6	0.6	b	2.7	1.8	a	0.8	0.5		1.2	1.8		1.2	0.8		
60	3-Furaldehyde***	1458	MS[14]	0.02	0.00		0.02	0.00		0.02	0.01		0.02	0.01	a	0.01	0.00	b	0.02	0.01	a	0.02	0.01		0.02	0.02		0.01	0.00		
61	Benzaldehyde	1503	ST	2.2	1.7		2.2	1.6		2.3	1.5		1.3	1.0		0.7	0.6		0.7	0.6		1.5	1.1		2.2	1.9		1.8	1.0		m,j
62	5-Methyl-2-furfuraldehyde	1556	ST	0.13	0.16		0.09	0.05		0.40	0.21		0.10	0.05	b	0.06	0.06	b	0.29	0.21	a	0.07	0.03		0.14	0.14		0.12	0.07		
63	*trans*-2-Decenal*	1636	MS[16]	0.02	0.01	B	0.03	0.02		0.02	0.01	**B**	0.04	0.01	a,b,B	0.02	0.01	b	0.06	0.03	a**,A**	0.08	0.04		0.08	0.04	A	0.05	0.03		c
64	Cuminaldehyde**	1779	MS[14]	0.02	0.01		0.01	0.00		0.02	0.02		0.02	0.02		0.01	0.01		0.04	0.06		0.01	0.00		0.02	0.02		0.02	0.01		
65	5-Hydroxymethylfurfural	2480	ST	0.02	0.01	B	0.01	0.01	*B*	0.03	0.01	**B**	0.06	0.01	A	0.07	0.03	*A*	0.08	0.03	**A**	0.02	0.00		0.03	0.01	B	0.02	0.01	*B*	j
66	α-Hexylcinnamaldehyde	2526	MS[14]	0.02	0.01		0.02	0.00	*A*	0.03	0.01		0.02	0.01	a	0.01	0.01	b,*B*	0.03	0.01	a	0.02	0.01		0.03	0.03		0.01	0.01	*AB*	
	Total of aldehydes			3.6	2.3		3.2	1.9		5	3		3.2	2.2		1.5	1.3		4	3		2.7	1.8		4	4		3.4	2.0		
	Ethyl esters																																			
67	Ethyl propionate	932	ST	0.24	0.12		0.5	0.5		0.39	0.23		0.2	0.3		0.4	0.5		0.8	0.6		0.18	0.10		1.2	2.2		1.3	1.5		
68	Ethyl 2-methylpropanoate	944	ST	0.23	0.12		0.5	0.6		0.34	0.12		0.15	0.20	b	0.3	0.3	a,b	1.0	0.9	a	0.33	0.21		2	3		0.9	0.9		
69	Ethyl butanoate	993	ST	0.10	0.07		0.24	0.24		0.14	0.04		0.11	0.15		0.3	0.4		0.4	0.3		0.08	0.04		3	6		3.49	6.35		
70	Ethyl 3-methylbutanoate	1024	ST	1.1	1.0		3	4		2.6	1.7		1.2	1.3	b	2.2	2.1	b	7	6	a	2.2	1.5		8	14		3.9	3.5		

71	Ethyl pentanoate	1093	ST	0.04	0.01		0.07	0.06		0.08	0.06		0.01	0.01	b	0.02	0.01	a,b	0.05	0.04	a	0.01	0.00		0.09	0.17		0.12	0.23						
72	Ethyl hexanoate	1207	ST	0.12	0.06		0.3	0.3		0.26	0.14		0.2	0.3	b	0.3	0.3	a,b	1.1	1.0	a	0.24	0.22		0.8	1.4		0.8	1.3						
73	Ethyl 3-hexenoate*	1273	MS[3]	0.02	0.00		0.02	0.01		0.03	0.01		0.01	0.01	b	0.01	0.01	b	0.05	0.03	a	0.02	0.02		0.04	0.06		0.03	0.04						
74	Ethyl heptanoate	1308	ST	0.00	0.00		0.03	0.04		0.01	0.01		0.01	0.01	b	0.01	0.01	b	0.04	0.03	a	0.00	0.00		0.03	0.06		0.03	0.04						
75	Ethyl 3-ethoxypropanoate*	1320	MS[19]	0.05	0.02	A	0.04	0.03		0.11	0.07	**A**	0.01	0.01	b,B	0.01	0.01	b	0.03	0.02	a,**B**	0.00	0.00		0.02	0.04	AB	0.02	0.03		m				
76	Ethyl 2-hydroxy-3-methylbutanoate*	1407	MS[14]	0.02	0.01		0.04	0.05		0.03	0.01		0.03	0.04		0.03	0.06		0.09	0.11		0.01	0.01		0.03	0.04		0.03	0.04						
77	Ethyl octanoate	1413	ST	0.11	0.09		0.10	0.04		0.23	0.20		0.13	0.23		0.18	0.19		0.6	0.6		0.20	0.17		1.1	2.0		1.0	1.8						
78	Ethyl 2-hydroxy-4-methylpentanoate*	1518	MS[12]	0.02	0.01		0.04	0.03		0.03	0.02		0.03	0.04		0.04	0.05		0.09	0.10		0.01	0.01		0.04	0.06		0.04	0.06						
79	Ethyl 2-furoate	1599	ST	0.04	0.02		0.05	0.03		0.06	0.06		0.07	0.09	a,b	0.05	0.02	b	0.20	0.15	a	0.02	0.01		0.10	0.14		0.10	0.08						
80	Ethyl decanoate	1618	ST	0.03	0.03		0.01	0.01		0.06	0.05		0.02	0.03		0.03	0.03		0.08	0.07		0.03	0.01		0.54	1.02		0.22	0.24						
81	Ethyl benzoate	1645	ST	0.04	0.01		0.03	0.01		0.07	0.07		0.08	0.15		0.05	0.03		0.14	0.15		0.02	0.01		0.11	0.15		0.12	0.13						
82	Diethyl succinate	1662	ST	0.04	0.04		0.15	0.19		0.10	0.06		0.1	0.3		0.2	0.3		0.5	0.8		0.04	0.04		0.2	0.4		0.6	1.3						
83	Ethyl phenylacetate	1767	ST	0.15	0.11	b	1.3	1.6	a	0.4	0.3	a,b, **B**	0.4	0.6	b	0.5	0.4	b	1.7	1.3	a,**A**	0.4	0.3		1.4	2.5		1.2	1.4						
84	Ethyl dodecanoate	1822	ST	0.01	0.00		0.01	0.00		0.02	0.02		0.01	0.00	b	0.00	0.00	b	0.01	0.00	a	0.01	0.00		0.07	0.11		0.02	0.02						
85	Ethyl hexadecanoate	2239	ST	0.01	0.00		0.02	0.01		0.01	0.01		0.01	0.00	b	0.01	0.00	b	0.02	0.01	a	0.01	0.01		0.02	0.02		0.01	0.01						
	Total of ethyl esters			2.38	1.73		6.78	7.55		4.91	3.15		2.74	3.74		4.49	4.77		14.1	11.8		3.75	2.66		18.2	32.9		13.85	18.90						
	Methyl esters																																		
86	Methyl nonanoate	1469	MS[14]	0.00	0.00	B	0.00	0.00		0.00	0.00		0.00	0.00	a,b,B	0.00	0.00	b	0.01	0.01	a	0.06	0.06		0.03	0.03	A	0.06	0.07		c				
87	Methyl decanoate*	1572	ST	0.02	0.01	a,b	0.04	0.00	a,*A*	0.01	0.00	b	0.03	0.03		0.00	0.00	*B*	0.04	0.04		0.01	0.01		0.02	0.02		0.01	0.01	*B*					

88	Methyl benzeneacetate***	1741	ST	0.02	0.01		0.02	0.01	*A*	0.03	0.01		0.01	0.01	a,b	0.00	0.00	b,*B*	0.02	0.01	a	0.01	0.00		0.03	0.02		0.01	0.01	*B*	m
89	Methyl salicylate	1754	ST	0.04	0.02		0.01	0.00		0.12	0.15		0.07	0.05		0.04	0.03		0.09	0.06		0.01	0.00		0.03	0.03		0.02	0.01		
90	Methyl 10-methylundecanoate	1779	DB	0.01	0.01		0.02	0.00		0.01	0.01		0.01	0.01	a,b	0.00	0.00	b	0.02	0.02	a	0.01	0.01		0.01	0.01		0.01	0.02		
91	Methyl tetradecanoate	1990	MS[14]	0.02	0.01		0.06	0.04	*A*	0.03	0.03		0.03	0.02	a,b	0.00	0.00	b,*B*	0.04	0.04	a	0.02	0.01		0.02	0.01		0.02	0.02	*B*	
92	Methyl pentadecanoate	2095	MS[14]	0.02	0.01		0.04	0.02	*A*	0.02	0.01		0.01	0.00	b	0.01	0.00	b,*B*	0.03	0.01	a	0.01	0.01		0.01	0.01		0.01	0.02	*B*	
93	Methyl hexadecanoate*	2200	MS[4]	0.02	0.01	B	0.01	0.00	*B*	0.02	0.01	**B**	0.09	0.05	a,b,AB	0.02	0.02	b,*B*	0.17	0.10	a,**A**	0.09	0.06		0.10	0.07	A	0.12	0.06	*A*	m
94	Methyl *cis*-9-hexadecenoate	2219	MS[14]	0.02	0.01		0.04	0.02	*A*	0.02	0.02		0.03	0.02	a,b	0.01	0.00	b,*B*	0.05	0.03	a	0.02	0.01		0.02	0.02		0.02	0.01	*B*	
95	Methyl 3-oxo-2-pentyl-cyclopentaneacetate	2287	MS[14]	0.04	0.01		0.04	0.01	*A*	0.08	0.04		0.05	0.02	b	0.01	0.01	c,*B*	0.08	0.03	a	0.05	0.02		0.06	0.04		0.03	0.01	*AB*	
96	Methyl octadecanoate	2402	MS[14]	0.01	0.01	b	0.17	0.17	a,*A*	0.06	0.06	a,b	0.05	0.03		0.01	0.01	B	0.05	0.04		0.02	0.02		0.01	0.01		0.03	0.02	*B*	
97	Methyl 9-octadecanoate*	2415	MS[14]	0.05	0.03		0.05	0.05	*A*	0.03	0.02		0.02	0.01	b	0.01	0.01	b,*B*	0.05	0.02	a	0.02	0.01		0.03	0.03		0.02	0.02	*B*	m,c
	Total of methyl esters			0.28	0.14		0.5	0.3		0.4	0.4		0.39	0.25		0.13	0.09		0.6	0.4		0.33	0.23		0.4	0.3		0.4	0.3		
	Ketones																																
98	Diacetyl	948	ST	2.7	1.3		1.6	0.9		2.51	1.15	**A**	1.1	0.4	a	0.5	0.4	b	0.7	0.3	a,b,**B**	0.42	0.08		1.4	1.6		1.1	0.8		m
99	3-Heptanone*	1135	ST	0.04	0.02	a,b,A	0.2	0.3	a,*A*	0.03	0.00	b,**A**	nd	nd	B	nd	nd	*B*	nd	nd	**B**	nd	nd		nd	nd	B	nd	nd	*B*	m
100	2,6-Dimethyl-4-heptanone*	1137	ST	0.03	0.01		0.04	0.00		0.03	0.01		0.02	0.00		0.03	0.01		0.03	0.01		0.03	0.00		0.04	0.03		0.03	0.01		
101	2-Heptanone*	1187	ST	0.04	0.01	A	0.09	0.08	*A*	0.05	0.01	**A**	nd	nd	B	nd	nd	*B*	nd	nd	**B**	nd	nd		nd	nd	B	nd	nd	*B*	m
102	Acetoin	1275	ST	1.5	0.9		0.4	0.3		1.4	0.9		0.60	0.21	a	0.30	0.15	b	0.80	0.18	a	0.32	0.10		0.8	0.5		0.	0.3		m
103	1-Hydroxy-2-propanone	1279	ST	0.32	0.13	a,A	0.03	0.02	b	0.17	0.04	a,b	0.12	0.06	a,b,B	0.04	0.02	b	0.4	0.4	a	0.17	0.07	a,b	0.34	0.16	a,A	0.13	0.10	b	
104	2,2,6-Trimethyl-cyclohexanone*	1292	MS[14]	0.01	0.00	B	0.00	0.00	*B*	0.02	0.02		0.01	0.00	a,b,B	0.00	0.00	b,*B*	0.01	0.01	a	0.10	0.03		0.3	0.3	A	0.17	0.10	*A*	c

105	6-Methyl-5-hepten-2-one***	1314	ST	0.03	0.01		0.03	0.00	*A*	0.05	0.04		0.03	0.01	b	0.01	0.00	b,*B*	0.06	0.03	a	0.03	0.02		0.04	0.03		0.02	0.01	*AB*	
106	3-Nonanone	1334	ST	0.01	0.00		0.03	0.02		0.02	0.01		0.01	0.01	b	0.01	0.00	b	0.04	0.03	a	0.02	0.01		0.07	0.11		0.06	0.08		
107	2-Cyclopentenone***	1340	MS[2]	0.01	0.00	B	0.01	0.00		0.01	0.01	**B**	0.04	0.01	a,A	0.02	0.01	b	0.05	0.02	a,**A**	0.04	0.01		0.04	0.03	A	0.03	0.01		m
108	1-Hydroxy-2-butanone***	1357	MS[21]	0.02	0.01	a,AB	0.01	0.00	b	0.02	0.00	a,b	0.02	0.00	b,B	0.01	0.00	b	0.04	0.03	a	0.02	0.01	a,b	0.03	0.01	a,A	0.01	0.01	b	
109	2-Nonanone*	1368	ST	0.04	0.02		0.06	0.04		0.05	0.02		0.03	0.01	b	0.03	0.01	b	0.07	0.04	a	0.06	0.05		0.16	0.22		0.14	0.16		
110	2-Acetylfuran	1487	ST	0.15	0.09		0.14	0.02	*A*	0.31	0.17		0.16	0.06	b	0.07	0.03	c,*B*	0.29	0.07	a	0.08	0.02		0.15	0.13		0.13	0.05	*AB*	m,c
111	2-Methyl-2-nonen-4-one	1526	DB	0.02	0.01	A	0.01	0.00	*B*	0.03	0.03		0.02	0.01	b,A	0.01	0.00	b,*A*	0.04	0.02	a	nd	nd		nd	nd	B	nd	nd	*C*	c
112	3,4-Dihydroxy-3,4-dimethyl- 2,5-hexanedione	1570	DB	0.18	0.08	A	0.12	0.03	*A*	0.14	0.02	**A**	0.05	0.01	b,B	0.02	0.01	b,*B*	0.08	0.03	a,**B**	0.07	0.03		0.15	0.08	A	0.09	0.06	*AB*	m,j,c
113	3,5,5-Trimethyl-2-cyclopenten-1-one	1601	MS	0.02	0.01		0.02	0.00		0.03	0.02	**B**	0.03	0.01	b	0.01	0.01	b	0.08	0.03	a,**A**	0.01	0.00		0.03	0.02		0.03	0.02		
114	Acetophenone**	1633	ST	0.11	0.04		0.07	0.01		0.11	0.10		0.09	0.04		0.06	0.04		0.14	0.09		0.06	0.02		0.12	0.10		0.13	0.07		
115	3-Methyl-2-pentyl-cyclopentanone	1636	DB	0.01	0.01		0.01	0.01	*B*	0.03	0.01		0.02	0.02		0.01	0.01	*B*	0.04	0.03		0.06	0.06		0.13	0.23		0.11	0.18	*A*	
116	2-Hydroxy-2-cyclopenten-1-one**	1768	MS[1]	0.06	0.02	a,B	0.01	0.00	b	0.06	0.01	a	0.02	0.01	b,C	0.00	0.00	b	0.05	0.03	a	0.05	0.01	b	0.10	0.04	a,A	0.03	0.01	b	j
117	Cyclotene	1833	ST	0.03	0.01	a,AB	0.01	0.01	b,*B*	0.03	0.00	a	0.02	0.00	b,B	0.00	0.00	c,*B*	0.04	0.02	a	0.04	0.01	a,b	0.07	0.05	a,A	0.03	0.01	b,*A*	j,c
118	1-(4-Methoxyphenyl)-1-hexanone	1834	DB	0.01	0.00		0.02	0.00	*AB*	0.02	0.01		0.01	0.00	a	0.01	0.00	b,*B*	0.02	0.01	a	0.03	0.01		0.06	0.06		0.03	0.02	*A*	c
119	4-Methyl-5H-furan-2-one	1892	DB	0.03	0.01	B	0.03	0.01	*AB*	0.04	0.01		0.03	0.01	a,b,B	0.01	0.01	b,*B*	0.04	0.01	a	0.05	0.01		0.08	0.06	A	0.04	0.02	*A*	j,c
120	1-(2,6,6-Trimethyl-1-cyclohexen-1-yl)-1-penten-3-one	2051	DB	0.05	0.03		0.07	0.01		0.08	0.04		0.06	0.04	a,b	0.02	0.02	b	0.07	0.03	a	0.08	0.03		0.10	0.08		0.07	0.04		
121	Benzophenone**	2455	ST	0.01	0.01		0.02	0.00	*A*	0.02	0.01	**A**	0.01	0.00	a	0.00	0.00	b,*B*	0.00	0.00	b,**B**	0.02	0.01		0.02	0.01		0.01	0.01	*AB*	j
	Total of ketones			6	3		3.1	1.7		5	3		2.5	1.0		1.2	0.8		3.1	1.4		1.7	0.6		4.1	3.7		2.9	2.1		***m***

	Lactones																														
122	δ-Decalactone**	1408	MS[8]	0.01	0.00		0.01	0.01		0.01	0.01	**B**	0.02	0.01	b	0.01	0.01	b	0.04	0.03	a,**A**	0.02	0.01		0.04	0.04		0.02	0.02			
123	γ-Butyrolactone**	1619	ST	0.08	0.04		0.06	0.03		0.04	0.00	**B**	0.07	0.02	a,b	0.04	0.02	b	0.11	0.05	a,**A**	0.09	0.03		0.12	0.06		0.06	0.02			
124	γ-Crotonolactone	1745	ST	0.11	0.03	a	0.04	0.02	b	0.09	0.03	a,b	0.07	0.02	b	0.03	0.02	b	0.14	0.08	a	0.09	0.03		0.12	0.07		0.06	0.03			
125	*trans*-β-Methyl-γ-octalactone	1884	MS[14]	0.04	0.04		0.02	0.00		0.03	0.03		0.02	0.01		0.01	0.01		0.05	0.06		0.01	0.00		0.04	0.04		0.03	0.02			
126	*cis*-β-Methyl-γ-octalactone	1956	ST	0.08	0.08		0.10	0.02		0.07	0.06		0.05	0.03		0.04	0.02		0.14	0.17		0.01	0.01		0.15	0.17		0.08	0.04			
127	γ-Nonalactone	2030	MS[9]	0.03	0.01		0.05	0.01		0.05	0.02		0.04	0.02	b	0.02	0.01	b	0.08	0.02	a	0.05	0.02		0.07	0.05		0.04	0.02			
128	5-Hydroxymethyldihydrofuran-2-one	2473	DB	0.05	0.02	a	0.01	0.00	b	0.03	0.01	a,b	0.03	0.01	b	0.01	0.00	b	0.06	0.03	a	0.04	0.01	a,b	0.05	0.03	a	0.02	0.01	b		
	Total of lactones			0.40	0.22		0.28	0.09		0.32	0.16		0.29	0.11		0.17	0.09		0.6	0.4		0.30	0.10		0.6	0.5		0.31	0.16			
	C_{13}-Norisoprenoids																														
129	1,1,6-Trimethyl-1,2-dihydronaphthalene	1719	ST	0.6	0.9		0.02	0.02		1.1	1.2		0.1	0.3		0.04	0.06		0.3	0.4		0.4	0.5		0.4	0.2		0.16	0.23			m,j
130	β-Damascenone**	1804	ST	0.02	0.01	a,b	0.01	0.00	b	0.04	0.02	a,**B**	0.05	0.02	b	0.02	0.01	c	0.09	0.03	a,**A**	0.02	0.00		0.04	0.04		0.02	0.01			j
	Total of C_{13}-Norisoprenoids			0.6	0.9		0.03	0.02		1.1	1.2		0.2	0.3		0.07	0.07		0.4	0.4		0.5	0.5		0.48	0.25		0.17	0.24			***m,j***
	Other esters																														
131	sec-Butyl acetate	961	ST	0.33	0.16		0.19	0.05		0.26	0.12		0.24	0.09		0.4	0.7		0.20	0.07		0.25	0.11		0.6	0.6		0.46	0.17			
132	Isoamyl propionate	1147	MS[9]	0.09	0.08		0.05	0.02		0.08	0.02		0.07	0.03		0.07	0.04		0.13	0.07		0.08	0.03		0.17	0.25		0.14	0.14			
133	Isoamyl 3-methylbutanoate	1270	MS[14]	0.06	0.03		0.03	0.00		0.07	0.02		0.04	0.03		0.04	0.04		0.10	0.07		0.11	0.09		0.2	0.3		0.11	0.11			
134	2-Hydroxyethyl propanoate**	1324	ST	0.05	0.02		0.05	0.04		0.11	0.07		0.10	0.17		0.12	0.20		0.6	1.0		0.08	0.06		0.3	0.6		0.19	0.22			
135	Isopropyl dodecanoate	1814	MS[14]	0.04	0.03		0.01	0.00		0.05	0.03		0.04	0.02	a,b	0.01	0.01	b	0.07	0.04	a	0.03	0.00	a,b	0.04	0.02	a	0.02	0.01	b		
136	Isopropyl tetradecanoate	2019	MS[14]	0.09	0.03		0.06	0.02	*A*	0.08	0.04		0.06	0.02	b	0.02	0.01	b,*B*	0.29	0.23	a	0.06	0.02		0.08	0.04		0.04	0.01	*AB*		

137	2-Phenylethyl pentanoate	2034	MS[14]	0.02	0.01		0.03	0.00		0.02	0.01		0.02	0.01		0.01	0.01		0.02	0.01		0.03	0.02		0.03	0.02		0.02	0.01		
138	n-Hexyl salicylate	2198	MS[7]	0.03	0.01		0.03	0.01		0.05	0.02		0.03	0.01	a	0.01	0.01	b	0.04	0.01	a	0.05	0.01		0.06	0.04		0.03	0.01		j,c
139	Isopropyl hexadecanoate	2223	MS[14]	0.02	0.01		0.03	0.01	*A*	0.03	0.01		0.03	0.01	a,b	0.01	0.01	b,*B*	0.05	0.03	a	0.03	0.02		0.03	0.02		0.02	0.01	*AB*	
	Total of other esters			0.73	0.39		0.48	0.16		0.75	0.32		0.62	0.39		0.68	1.04		1.52	1.50		0.70	0.36		1.47	1.91		1.04	0.71		
	Volatile phenols																																
140	Guaiacol**	1849	ST	0.04	0.01	A	0.03	0.01	*A*	0.04	0.01		0.04	0.01	b,A	0.02	0.01	c,*AB*	0.06	0.02	a	0.01	0.00		0.01	0.01	B	0.01	0.01	*B*	c
141	4-Ethylguaiacol	2020	ST	0.05	0.02		0.04	0.00		0.8	1.0		0.11	0.06		0.06	0.05		0.14	0.07		0.03	0.01		0.04	0.02		0.04	0.03		
142	*p*-Cresol***	2083	ST	0.05	0.01	A	0.04	0.01	*A*	0.05	0.03		0.02	0.01	b,B	0.01	0.00	c,*B*	0.04	0.01	a	0.02	0.00		0.02	0.02	B	0.01	0.01	*B*	m
143	Eugenol**	2163	ST	0.01	0.00		0.00	0.00		0.01	0.00		0.01	0.01	a	0.00	0.00	b	0.01	0.01	a	0.00	0.00		0.01	0.01		0.00	0.00		
144	4-Ethylphenol**	2177	ST	0.06	0.04	B	0.06	0.00		0.09	0.05	**B**	0.16	0.07	a,b,A	0.08	0.05	b	0.22	0.09	a,**A**	0.06	0.05		0.07	0.05	B	0.11	0.13		m,j
145	Coumaran**	2400	MS[19]	0.02	0.01		0.01	0.00	*A*	0.03	0.02		0.03	0.01	a	0.00	0.00	b,*B*	0.03	0.02	a	0.02	0.01	a,b	0.03	0.03	a	0.01	0.00	b,*AB*	
	Total of volatile phenols			0.22	0.09		0.18	0.03		1.0	1.1		0.38	0.16		0.18	0.11		0.50	0.22		0.14	0.07		0.19	0.14		0.18	0.18		***m,j***
	Terpenes																																
146	Camphene	1060	MS[14]	0.12	0.08	a,b	0.03	0.02	b	0.27	0.14	a	0.07	0.09		0.04	0.03		0.14	0.12		0.05	0.02		0.05	0.03		0.03	0.03		m
147	Eucalyptol**	1177	ST	0.01	0.01	B	0.01	0.00		0.02	0.02		0.01	0.00	b,B	0.00	0.00	b	0.01	0.00	a	0.05	0.03		0.05	0.04	A	0.04	0.04		c
148	*trans*-p-Mentha-2,8-dienol	1304	DB	0.01	0.00	AB	0.00	0.00		0.02	0.01		0.00	0.00	b,B	0.00	0.00	b	0.01	0.00	a	0.01	0.01		0.01	0.01	A	0.01	0.01		j
149	Linalool oxide**	1431	ST	0.06	0.02		0.07	0.03		0.11	0.05		0.08	0.04	a,b	0.04	0.02	b	0.13	0.06	a	0.07	0.03		0.09	0.08		0.08	0.06		
150	Dihydromyrcenol*	1462	MS[17]	0.01	0.01		0.02	0.00		0.01	0.01		0.02	0.01	a	0.01	0.01	b	0.01	0.01	a,b	0.02	0.01		0.04	0.03		0.01	0.01		
151	Safranal **	1627	ST	0.01	0.01		0.01	0.00		0.02	0.01		0.04	0.02		0.02	0.02		0.05	0.02		0.05	0.01		0.04	0.04		0.04	0.03		m
152	α-Terpineol **	1704	ST	0.01	0.00	B	0.01	0.00		0.02	0.01		0.01	0.00	a,b,B	0.01	0.00	b	0.01	0.00	a	0.03	0.01		0.04	0.03	A	0.03	0.02		c

153	Dihydropseudoionone*	1838	MS[14]	0.08	0.03		0.08	0.00	*A*	0.11	0.05		0.06	0.02	b	0.02	0.01	b,*B*	0.11	0.06	a	0.09	0.04		0.11	0.08		0.06	0.02	*A*	
	Total of terpenes			0.31	0.16		0.23	0.06		0.6	0.3		0.29	0.18		0.13	0.09		0.5	0.3		0.36	0.15		0.4	0.3		0.30	0.22			
	Miscellaneous																																
154	Pyrrole	1487	MS[18]	0.08	0.09		0.02	0.01		0.05	0.02	**A**	nd	nd		nd	nd		nd	nd	**B**	0.16	0.15		0.08	0.08		0.4	0.6			j,c
155	Acetamide **	1787	MS[14]	0.05	0.01		0.05	0.03		0.05	0.03		0.05	0.01	a,b	0.03	0.02	b	0.10	0.08	a	0.06	0.02		0.08	0.07		0.05	0.02			
156	2H-Pyran, tetrahydro-2-(12-pentadecynyloxy)-	1808	DB	0.04	0.01	a,b,A	0.02	0.01	b	0.05	0.02	a	0.04	0.01	a,b,A	0.02	0.01	b	0.05	0.02	a	0.02	0.01		0.02	0.01	B	0.01	0.01			c
157	n.i. (m/z 85,100)	1917	MS[14]	0.06	0.01	AB	0.05	0.02	*A*	0.08	0.04		0.08	0.04	b,A	0.03	0.02	b,*A B*	0.13	0.05	a	0.02	0.01		0.02	0.03	B	0.01	0.01	*B*		c
158	Benzothiazole	1950	MS[13]	0.02	0.00	A	0.02	0.00	*A*	0.02	0.02	**A**	nd	nd	B	nd	nd	B	nd	nd	**B**	nd	nd		nd	nd	B	nd	nd	*B*		m
159	n.i. (m/z 55,69,83)	2064	DB	0.03	0.01	B	0.02	0.00	*B*	0.03	0.01	**B**	0.04	0.01	a,b,B	0.02	0.01	b,*B*	0.06	0.02	a,**A**	0.08	0.02		0.12	0.10	A	0.06	0.02	*A*		c
160	n.i. (m/z 85,29,41)	2085	DB	0.06	0.02	A	0.05	0.02	*A*	0.06	0.03		0.03	0.01	b,B	0.01	0.01	b,*B*	0.05	0.02	a	0.01	0.01		0.02	0.01	B	0.01	0.00	*B*		m,j, c
	Total of Miscellaneous			0.34	0.15		0.23	0.09		0.35	0.16		0.24	0.08		0.12	0.07		0.40	0.19		0.35	0.21		0.4	0.3		0.5	0.6			

[1]: *detected in product derived from grape and for the first time in vinegars; **detected for the first time in *Vinagre de Condado de Huelva* and *Vinagre de Montilla-Moriles* PDOs; ***detected for the first time in *Vinagre de Jerez* PDO.

ID (identification): reliability of identification: ST, mass spectrum and LRI agreed with standards (own or literature); MS, mass spectrum agreed with mass spectral data base and LRI agreed with the literature data; DB, mass spectrum agreed with mass spectral data base. [2]: Number in superscript in this column correspond to the reference were the compounds has also been identified: 1: Antoniotti, Alezra, Fernandez & Dunach, 2004; 2: Chevance & Farmer, 1999; 3: Ferrari et al., 2004; 4: Hanai & Hong, 1989; 5: Liang, Chen, Reeves & Han, 2013; 6: Liu et al., 2017; 7: Lukic, Radeka, Grozaj, Staver, & Persuric, 2016; 8: Marrufo-Curtido et al., 2012; 9: Morales, Fierro-Risco, Callejón, & Paneque, 2017; 10: Ohatah, Tominaga, Dubourdieu, Kubota, & Sugawara, 2009; 11: Peña, Barciela, Herrero, & García-Martín, 2005; 12: Pino & Queris, 2011; 13: Pozo-Bayon, Ruiz-Rodriguez, Pernin,& Cayot, 2007; 14: Pubchem database 2005; 15: Ruiz-Bejarano, Castro-Mejías, Rodríguez-Dodero & García-Barroso, 2013; 16: Rychlik, Schieberle & Grosch, 1998; 17: Sánchez-Palomo, Alañón, Díaz-Maroto, González-Viñas & Pérez-Coello, 2009; 18: Shimoda, Shigematsu, Shiratsuchi & Osajima, 1995; 19: Ubeda, et al., 2016; 20: Versari, Laurie, Ricci, Laghi, & Parpinello, 2014. 21: Wang, Lin, Song & Yao, 2010. (Complete references below). RA: Mean relative areas. SD: Standard deviation. S: Significant differences.

S[3]: Different lowercase letters in different columns indicate significant differences according to Tukey's test ($p<0.05$) between categories within each PDO. Different capital letters in different columns indicate significant differences according to Tukey's test ($p<0.05$) between similar categories of different PDOs: A, B, C = Significant difference between JCR, MCR and CSO; *A, B, C* = Significant difference between JRE, MRE and CRE; **A, B** = Significant difference between JPX and MPX.

TT 4: Tukey's test ($p<0.05$) among PDOs. Letters indicate: m,j= significant difference between VJ and VM; j,c= significant difference between VJ and VC; m,c = significant difference between VC and VM; m,j,c= significant difference between all PDOs; j=significant difference between VJ and the others; c=significant difference between VC and the others; m=significant difference between VM and the others. nd: Peak not detected; n.i: non identified peak.

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BLOQUE III.

CARACTERIZACIÓN Y CLASIFICACIÓN SENSORIAL DE LOS VINAGRES DE VINO ESPAÑOLES CON DOP

CAPÍTULO VII:
Caracterización del perfil aromático de los vinagres de vino españoles con DOP

CHAPTER VII.

Characterization of the aromatic profile of Spanish PDO wine vinegars

RESUMEN

Tras haber realizado el análisis de la composición volátil de los vinagres de vino españoles con DOP, de y sus categorías, es necesario estudiar y determinar cuáles son los compuestos volátiles que contribuyen principalmente al aroma general percibido de la muestra, denominados odorantes de impacto. Este estudio es necesario y relevante ya que el aroma es uno de los indicadores de calidad más importantes para los vinagres de vino de alta calidad.

Por tanto, en este capítulo de tesis se realizó, por primera vez, la caracterización aromática de los vinagres de vino españoles con DOP, y de sus dos categorías principales según producción y ventas (Reserva y Pedro Ximénez), mediante el análisis por cromatografía de gases-espectrometría de masas acoplado con olfatometría (GC-MS-O) y el análisis sensorial, con el objetivo de describir y comparar sus perfiles aromáticos, así como determinar sus correspondientes odorantes de impacto y su relación con los perfiles sensoriales. Este trabajo se encuentra enviado a Food Chemistry 2019.

Para realizar este estudio, se seleccionaron 3 muestras representativas de vinagres de vino de la categoría Reserva de las tres DOP españolas, así como 2 vinagres de vino de la categoría Pedro Ximénez de las DOP que lo incluyen en su reglamento (Vinagre de Jerez y Vinagre de Montilla-Moriles). Los aromas fueron extraídos por extracción Liquido-Liquido (ELL) y analizados por GC-MS-O. La técnica olfatométrica empleada fue la frecuencia modificada (FM). Así, los análisis olfatométricos fueron realizados por un panel de 3 catadores entrenados, los cuales tenían que registrar la descripción del olor y la intensidad percibida. Además, para la determinación y confirmación de los odorantes de impacto también se realizó la determinación del valor de la actividad del olor (OAV), es decir, la relación entre la concentración y el umbral del olor. Por otro lado, estas muestras también se analizaron mediante análisis sensorial empleando el análisis cuantitativo descriptivo (QDA).

Para la determinación de los umbrales olfativos, así como para realizar el análisis sensorial de las muestras, se empleó un panel de catadores entrenados, formado por 5 mujeres (en la que me incluyo) y 3 hombres, pertenecientes al departamento de Nutrición y Bromatología de la Facultad de Farmacia, US, así como de otros centros y universidades que se encontraban realizando estancias de investigación.

Del total de zonas odorantes detectadas en el estudio, se seleccionaron 103 zonas odorantes por su alta FM, las cuales, según su descriptor aromático, se agruparon en 9 categorías. Tras realizar un análisis de componentes principales (PCA) y un análisis de varianza

(ANOVA), los resultados mostraron como las muestras de la DOP Vinagre de Montilla-Moriles se caracterizaron por tener un importante número de odorantes de impacto con aromas lácteos, con algunos matices químicos para su categoría Reserva (MRE) y matices tostados, picantes y dulces para su categoría dulce (MPX); mientras que las muestras de la DOP Vinagre de Jerez mostraron un mayor porcentaje de odorantes de impacto con carácter herbáceo, para las muestras Reserva (JRE), seguida de matices picantes, dulces y florales, predominantes en las muestras Pedro Ximénez (JPX); y finalmente las muestras envejecidas de Vinagre de Condado de Huelva (CRE) mostraron una mayor presencia de caracteres químicos, seguidos de caracteres aromáticos picantes y afrutados. Además, la principal diferencia entre las muestras dulces de cada DOP fue que MPX presentó mayor porcentaje de odorantes de impacto con notas aromáticas dulces y tostadas que JPX, que a su vez presentó más porcentaje de odorantes de impacto con notas florales que MPX.

Por otro lado, una vez que se evaluó la contribución de cada compuesto volátil al aroma de los vinagres de vino mediante GC-MS-O, se realizó una evaluación cuantitativa de estos aromas de impacto mediante la media los OAVs. Los resultados de OAV confirmaron que los odorantes de impacto característicos para cada DOP y categoría (FM>80%) realmente contribuían al aroma de cada muestra debido a que presentaban OAVs>1. Así, los odorantes de impacto según FM y OAV seleccionados como marcadores fueron propionato de etilo, octanoato de etilo, ácido propanoico y 4-etilfenol para JRE ,junto con *cis*-2-nonenal y acetato de *cis*-3-hexenilo según sólo la FM; diacetilo y metional y furfrual para JPX, junto con 6,7-dihydro-7-hidroxilinalool según solo FM; acetoína para MRE, junto con abhexona según FM; acetato de etilfenilo y vainillina para MPX, junto con 2,3-butanedioldiacetato, 2,6-dimetilpirazina, dihidromaltol, ciclohexanoato de etilo, 3-nonen-2-ona, β-damascenona, p-vinilguaiacol y ácido benzoico; y acetaldehído dietilacetal, acetato de isobutilo, isovalerato de etilo y guaiacol para CRE, junto con etanol, acetato de etilo, 3-metil-1-butanol, salicilato de etilo, β-ionona y maltol, según sólo la FM.

Además, la evaluación sensorial de las muestras mostró patrones similares a los anteriormente obtenidos por GC-MS-O y OAVs: la puntuación para los descriptores de sensación punzante mostró diferencias significativas entre CRE, la cual alcanzó los mayores valores, y el resto, mientras que el descriptor de aroma a pasas destacó de manera significativa en los vinagres JPX y el aroma a dulce en los MPX. Cuando estos descriptores sensoriales se agruparon del mismo modo que los odorantes de impacto detectados por el análisis GC-O, se observaron resultados similares entre ellos, lo que reafirmó la fiabilidad y utilidad del análisis por GC-O para la caracterización aromática y diferenciación de estas muestras.

ARTÍCULO 10

Aroma profile and key odorants of Spanish PDO wine vinegars by gas chromatography-mass spectrometry-olfactometry (GC-MS-O), odour active values (OAVs) and sensory analysis (QDA)

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Abstract: The aroma profile of Spanish wine vinegars with Protected Designation of Origin (PDO) were described and compared for the first time by gas chromatography-mass spectrometry-olfactometry (GC-MS-O), odour active values (OAVs) and quantitative descriptive analysis (QDA). Vinagre de Jerez Reserva (JRE) showed higher percentage of 'grassy-vegetal' impact odorants, while the "spicy" ones highlighted for Pedro Ximénez category (JPX). Vinagre de Montilla-Moriles Reserva (MRE) had a large 'buttery-lactic' impact odorants, while the 'empyreumatic' and 'sweet' ones stood out for Pedro Ximénez category (MPX). Vinagre de Condado de Huelva Reserva (CRE) showed a stronger percentage of 'chemical' impact odorants. The key odorants were ethyl propionate, ethyl octanoate, propanoic acid and 4-ethylphenol for JRE, diacetyl and methional-furfural for JPX, acetoin for MRE, ethyl phenylacetate and vanillin for MPX and acetaldehyde diethyl acetal, isobutyl acetate, ethyl isovalerate and guaiacol for CRE. A good relation among the impact odorants and the sensory descriptors was observed.

***Editor-in-Chief* P. Finglas,**
Quadram Institute Bioscience,
Norwich, England, UK
Seville, 4th June 2019

Dear *Editor-in-Chief* P. Finglas,

Enclosed please you find a softcopy of the manuscript entitled " **AROMA PROFILE AND KEY ODORANTS OF SPANISH PDO WINE VINEGARS BY GAS CROMATHOGRAPHY-MASS SPECTROMETRY-OLFACTOMETRY (GC-MS-O), ODOUR ACTIVE VALUES (OAVs) AND SENSORY ANALYSIS (QDA)"** by Rocío Ríos-Reina (rrios5@us.es), Pilar Segura-Borrego (pilar_sb94@hotmail.com), M Lourdes Morales (mlmorales@us.es) and Raquel M. Callejón (rcallejon@us.es), for publication in Food Chemistry.

Currently, Spain plays an important role in the production of wine vinegars of high quality that have being registered with a PDO: Vinagre de Jerez, Vinagre de Montilla-Moriles and Vinagre de Condado de Huelva. Furthermore, within each PDO, there are different categories according to their time and type of ageing in wooden barrels, as well as other characteristics of production. The combination of growing consumer demand, the increasing diversity of wine vinegars, and the high quality of these PDO wine vinegars have created the need to characterise them and to establish an adequate quality control in order both to defend their identity and to combat fraud. The age of the vinegars and their semi-sweet property has a remarkable impact on the vinegars' aroma and is the reason for their high price. Aroma is, therefore, one of the main quality indicators of vinegars and it depends on the volatile compounds profile. The vinegar's volatile fraction contains a large number of compounds, yet of all of these compounds, only some volatile compounds, known as odour-active compounds, contribute to its overall aroma and play an important role in aroma perception. In particular, it is the impact odorants that directly provide their specific aromatic characteristics to the perceived aroma.

In this context, the objective of this work was three-fold: firstly, to describe and compare for the first time the aroma profile of Spanish wine vinegars with Protected Designation of Origin by gas chromatography-mass spectrometry-olfactometry (GC-MS-O), using 'modified frequency' technique (MF), odour active values (OAVs) and quantitative descriptive analysis (QDA); secondly, to determine their characteristics impact odorants, which could be considered as quality and authenticity markers for these vinegars; and

thirdly to study the relation among the impact odorants and to determine which odorants and sensory attributes could differentiate between PDOs and categories. The results showed that some differences were observed regarding the aromatic profile of each PDO and category (*Reserva* and *Pedro Ximénez*) due to the presence of impact odorants that were shown to be characteristic for each kind of wine vinegar. Hence, these compounds could be selected as markers or key odorants useful for their discrimination and authentication.

In addition, all authors certify that they participated in the conception of the work and make public my responsibility for its content and that they did not omit any connections or funding agreements among the authors and companies that may have an interest in the publication of this article, and they approve the final manuscript which is submitting to Food Chemistry. All of them approve the authorship criteria and give to this journal the exclusive rights to edit, publish, reproduce, distribute copies, make ready derivative works in paper, electronic or multimedia and include the article in indexes or national and international databases.

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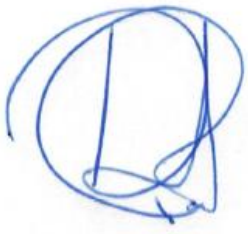
Best regards,



***Highlights (for review)**

- The aromatic profiles of two categories of PDO wine vinegars were studied.
- The analyses were performed by GC-MS-O, with MF technique, OAVs and a QDA.
- Some impact odorants were selected as markers for each PDO or category.
- The wine vinegars also showed differences in some sensory attributes.
- A good correlation between sensory descriptors and impact odorants was observed.

TITLE: AROMA PROFILE AND KEY ODORANTS OF SPANISH PDO WINE VINEGARS BY GAS CROMATHOGRAPHY-MASS SPECTROMETRY-OLFACTOMETRY (GC-MS-O), ODOUR ACTIVE VALUES (OAVs) AND SENSORY ANALYSIS (QDA)

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Abstract

The aroma profile of Spanish wine vinegars with Protected Designation of Origin (PDO) were described and compared for the first time by gas chromatography-mass spectrometry-olfactometry (GC-MS-O), odour active values (OAVs) and quantitative descriptive analysis (QDA). *Vinagre de Jerez Reserva* (JRE) showed higher percentage of 'grassy-vegetal' impact odorants, while the "spicy" ones highlighted for *Pedro Ximénez* category (JPX). *Vinagre de Montilla-Moriles Reserva* (MRE) had a large 'buttery-lactic' impact odorants, while the 'empyreumatic' and 'sweet' ones stood out for *Pedro Ximénez* category (MPX). *Vinagre de Condado de Huelva Reserva* (CRE) showed a stronger percentage of 'chemical' impact odorants. The key odorants were ethyl propionate, ethyl octanoate, propanoic acid and 4-ethylphenol for JRE, diacetyl and methional-furfural for JPX, acetoin for MRE, ethyl phenylacetate and vanillin for MPX and acetaldehyde diethyl acetal, isobutyl acetate, ethyl isovalerate and guaiacol for CRE. A good relation among the impact odorants and the sensory descriptors was observed.

Keywords: wine vinegar, Protected Designation of Origin, impact odorant, GC-MS-O, *Reserva*, *Pedro Ximénez*.

1. Introduction

In general, wine vinegar is the most highly-prized and the most commonly-used vinegar in Europe, especially in the Mediterranean basin. Spain plays an important role in the production of these high-quality wine vinegars. Hence, in the south of Spain, there are three wine vinegar marketed under a specific Protected Designation of Origin (PDO): *Vinagre de Jerez*, *Vinagre de Condado de Huelva* and *Vinagre de Montilla-Moriles* (Council Regulation (EC) No 510/2006).

These vinegars are obtained exclusively from the acetous fermentation of 'suitable wines', produced according to the specifications of their designations. One of the main differences between each PDO is the variety of grape used: mainly *Palomino* for *Vinagre de Jerez* PDO, *Pedro Ximénez* for *Vinagre de Montilla-Moriles* PDO and *Zalema* for *Vinagre de Condado de Huelva* PDO (BOJA, 03/10/08; BOJA, 16/09/08a; BOJA, 16/09/08b).

Moreover, different categories can be distinguished within each PDO, based upon the vinegars' different ageing periods using the traditional *Criaderas y solera* system: those with a minimum ageing period of six months are termed *Vinagre de Jerez*, *Vinagre Viejo Condado de Huelva Solera* and *Vinagre de Montilla-Moriles Crianza*, according to their PDO; vinegars with a minimum ageing period of two years are termed *Reserva* in the three PDOs. Finally, the vinegars which have undergone at least 10 years' aging are known as *Gran Reserva*. These are only produced in the *Vinagre de Jerez* and *Vinagre de Montilla-Moriles* PDOs (BOJA.03/10/08; BOJA.16/09/08a)

Vinagre de Jerez and *Vinagre de Montilla-Moriles* PDOs also include another type of vinegars grouped as 'sweet vinegars' –the *Vinagre al Pedro Ximénez* category and the least-produced category, *Vinagre al Moscatel*. Each PDO produces their sweet vinegars by different procedures. Hence, *Vinagre de Montilla-Moriles al Pedro Ximénez* is produced by adding must from sun-dried *Pedro Ximénez* grapes to the vinegar

during the ageing process, whereas *Vinagre de Jerez al Pedro Ximénez* is produced by adding *Pedro Ximénez* wines (obtained from sun-dried *Pedro Ximénez* grape must) from the same PDO to the vinegars during their ageing period.

Among all of these categories, the best-selling are mainly the *Reserva* and the *Pedro Ximénez* categories. These vinegars are of high quality due to their production procedures which provide them with unique characteristics that increase the perception of quality by the consumers. This perception is mainly based on consumers' perception of their aroma. Thus, the aging period of *Reserva* wine vinegars in wooden barrels produces changes in their aromatic profiles responsible for increasing the vinegar's aromatic complexity and, consequently, for increasing its quality(Callejón, Morales, Silva Ferreira, & Troncoso, 2008). Something similar occurs in the *Vinagre al Pedro Ximénez* category; adding must or wines from sun-dried grapes also produces a change in the vinegars' composition and hence in their aromatic characteristics, something highly appreciated by consumers.

The aroma of food and beverages is key for consumer acceptance. Hence, aroma has been described as one of the most important indicators of vinegar quality (Callejón, Morales, Silva Ferreira, et al., 2008; Chinnici et al., 2009). The vinegar's volatile fraction contains a large number of compounds, yet of all of these compounds, only some volatile compounds, known as odour-active compounds, contribute to its overall aroma and play an important role in aroma perception. In particular, it is the impact odorants that directly provide their specific aromatic characteristics to the perceived aroma.

On the one hand, gas chromatography-mass spectrometry coupled to olfactometry (GC-MS-O) has become an important and essential tool for characterizing the olfactory impact of the odorants (Mayol & Acree, 2001; Zellner, Dugo, Dugo, & Mondello, 2008). Hence, this technique enables researchers to clarify whether a volatile compound has an odour characteristic (i.e. odour descriptor) and thus evaluate its contribution to the

overall aroma (i.e. odour intensity) (Fischer & Hammerschmidt, 1992). Concerning GC-MS-O food flavour analysis, some factors must be borne in mind, such as the sample preparation. The choice of an appropriate sample preparation method becomes, therefore, a crucial step. In vinegar, the most widely-used sampling techniques coupled with GC-MS-O have been solid phase microextraction (SPME) and liquid-liquid extraction (LLE), although there are few works of research regarding this food matrix (Aceña, Vera, Guasch, Busto, & Mestres, 2011; Callejón, Morales, Silva Ferreira, et al., 2008; Callejón, Morales, Troncoso, & Silva Ferreira, 2008).

There are three different types of olfactometric techniques: dilution analysis, perceived intensity and frequency of detection. A combination of the intensity and frequency of detection techniques conforms a hybrid technique known as 'modified frequency' (MF). MF has been demonstrated to provide more reliable results than the others due to the fact that the discriminative capabilities of detection frequency are improved by taking intensity into account (Dravnieks, 1985). This methodology is based in the geometric mean of the detection frequency of an aromatic zone and the average intensity expressed as a percentage.

Moreover, not only is the screening of significant odorants undertaken GC-MS-O, but also by using the odour activity value (OAV) concept (i.e. ratio of concentration to odour threshold). OAV affirms that odorants should contribute to the overall aroma if they exceed their odour thresholds (OAV>1) in the matrix. OAV has been widely applied in determining the aroma compounds which most likely contribute to the overall odour of a food and could be used together with GC-MS-O analysis, complementing and improving the suitability of the results (Callejón, Morales, Troncoso, et al., 2008; Zellner et al., 2008).

Sensory analysis is also a valuable tool for evaluating the quality of vinegar from the point of view of the producer, researcher or consumer. The sensory profile of the traditional vinegars made in the South of Spain and protected under a PDO, has mainly

been associated with their aging in wood and their higher alcohol residue compared to other types of vinegars.

The aroma and the volatile composition of *Vinagre de Jerez* has been studied by several authors (Callejón, Morales, Silva Ferreira, et al., 2008; Marrufo-Curtido et al., 2012; M Lourdes Morales, Tesfaye, García-Parrilla, Casas, & Troncoso, 2002). However, the aroma of vinegars belonging to its sweet category, Pedro Ximénez wine vinegar, as well as vinegars from the *Vinagre de Condado de Huelva* and *Vinagre de Montilla-Moriles* PDOs have, to our knowledge, never been studied. Thus, the aim of this work was: i) to describe and compare the aromatic profiles of *Reserva* and *Pedro Ximénez* vinegars from the three different Spanish PDOs by GC-MS-O; ii) to determine their key odorants; iii) to study their relation to the sensory profiles; iv) and finally, to determine which odorants and sensory attributes could differentiate between PDOs and categories. Hence, in this work has for the first time, studies aromatic profiling performed by GC-MS-O of the *Reserva* vinegars from *Vinagre de Condado de Huelva* and *Vinagre de Montilla-Moriles* PDOs, and the *Pedro Ximénez* PDO vinegars.

2. Materials and methods

2.1. Vinegar Samples

Three representative 2-year-old vinegars (*Reserva* category) of each PDO (*Vinagre de Jerez* PDO: JRE, *Vinagre de Condado de Huelva* PDO: CRE and *Vinagre de Montilla-Moriles* PDO: MRE) were selected by an expert panel for sensory analysis as being a vinegar “type” for each PDO. Their acetic degree was between 7.5 and 8% (w/v). Moreover, two wine vinegars belonging to the sweet category of *Vinagre de Jerez* and *Vinagre de Montilla-Moriles* PDOs, termed *Vinagre al Pedro Ximénez*, were also included in the study (JPX and MPX, respectively). A *Pedro Ximénez* sample of the PDO *Vinagre de Condado de Huelva* was not studied because this category is not

106 included in the European regulation covering the *Vinagre de Condado de Huelva* PDO.
107 All samples were provided by the respective Regulatory Councils.

108 **2.2. Chemicals and Reagents**

109 The standards of the aroma compounds given in **Table 1** were obtained from the
110 commercial sources Sigma-Aldrich (Madrid, Spain) and Merck (Darmstadt, Germany).
111 4-Methyl-2-pentanol (Merck, Darmstadt, Germany) was employed as internal standard
112 (IS). Dichloromethane, anhydrous sodium sulphate, sodium chloride, and acetic acid
113 were obtained from Merck (Darmstadt, Germany) and all of them were of analytical
114 quality. Water was obtained from a Milli-Q purification system (Millipore, Billerica, MA).

115 **2.3. Sensory analysis**

116 ***2.3.1. Sensory panel***

117 The expert sensory panel performing the different tests described in this work
118 comprised eight panellists (five women and three men), all belonging to the laboratory
119 and with experience in the sensory analysis of wine vinegar. Training was performed
120 according to international protocols (ISO.4120, 1983; ISO.6658, 2009).

121 ***2.3.2. The quantitative descriptive analysis (QDA)***

122 A quantitative descriptive analysis was performed by the protocol established and
123 validated by Tesfaye et al., (2010). The sensory panelists were asked to score a series
124 of attributes that had been chosen by consensus by marking the intensity on an
125 unstructured 10 cm straight line labeled “not noticeable” and “very strong” on the left
126 and right end points, respectively (ISO.4121, 1987). These selected attributes marked
127 on the tasting cards were: pungent sensation, ethyl acetate, wine character, woody, red
128 fruits, sweet, bitter almond, vanilla, citric, licorice, leather-old and raisin. In addition, 6
129 other sensory attributes were included in the test (medicinal, coconut, green, bacteria,
130 cheese and sawdust), for which it was considered more practical to mark their

absence/presence rather than their intensities. These attributes' inclusion were considered as optional, as is the case with other authors (W. Tesfaye et al., 2010).

2.3.3. *Threshold Determination*

Among the different methodologies used to calculate thresholds for the volatile compounds available, this study selected and used the method approved by the American Society for Testing and Materials (ASTM).

First, an ascending order test was performed to delimit the proper concentration range to study and to familiarise panellists with the odour of the compounds. Five 2-fold dilutions (x/4, x/2, X, 2x and 4x) were prepared by diluting the substance whose threshold was going to be determined in the medium of interest (acetic acid 7% w/v) (Callejón, Morales, Silva Ferreira, et al., 2008). Panellists were asked to indicate the solution in which they perceived any odour different to pungent odour of the acetic acid. We fixed the X value (concentration of the volatile compound) as a concentration that was 2-fold higher than the corresponding threshold values referenced in the literature for wine and wine vinegar (Callejón, Morales, Silva Ferreira, et al., 2008; Charles et al., 2000; Chinnici et al., 2009)

Secondly, and according to Plotto et al., (2004), the three-alternative forced choice (3-AFC) test was employed for threshold determination (ASTM Designation: E-679, 2004). by using control samples (7% acetic acid solution) against test dilutions (standard in 7% acetic acid solution) (Plotto, Margaría, Goodner, Goodrich, & Baldwin, 2004). The test dilutions differed from the preceding one by a factor of 2 (2x, x, x/2, x/4...), and successive dilutions were tested until the lowest was consistently missed. The amount of the aromatic compound 2x corresponds to the minimum concentration of the substance that was perceived by at least 80% of the panel in the ascending order test. The best-estimate criterion was then used to calculate individual thresholds as follows: the threshold for each individual (best-estimate threshold) was an interpolated value

157 determined as the geometric mean of the values obtained from the square root of the
158 last concentration missed and the first concentration detected of each panellist. Finally,
159 the panel threshold of each substance was calculated as the geometric mean of the
160 best-estimate thresholds of every individual panellist for each compound.

161 ***2.3.4. GC-MS-O analysis***

162 The selected samples were submitted to a liquid–liquid extraction method (LLE) to
163 perform the GC-MS-O analysis. LLE was performed according to the methodology
164 validated by Ferreira et al. (2003) and used by the authors in previous works (Callejón,
165 Morales, Troncoso, et al., 2008). The dichloromethane extracts from the different
166 vinegars, with the addition of 10 µL of IS, were then analysed by GC-MS-O.

167 The gas chromatography analysis was performed with a 6890 Agilent GC system
168 coupled to a quadrupole mass spectrometer Agilent 5975inert and an olfactory
169 detection port (ODP3, Gerstel), with the following chromatographic conditions: The
170 columns used were a CPWax- 57CB, with 50 m × 0.25 mm and 0.20 µm film thickness
171 (Varian, Middelburg, Netherlands) and an HP5 column of 30 m x 0.25 mm and 0.25 µm
172 film thickness (Agilent). 5 µL of the extracted sample was injected into the injector port
173 heated to 220 °C in splitless mode for 1 min, with a total flow rate of 73.5 mL/min. The
174 oven temperature was 40 °C (for 1 min) and then increased by 2 °C/min to 220 °C and
175 held there for 30 min. The column effluent was split 1:1 into a mass spectroscopy
176 detector (MS) and an ODP by means of a GRAPHPACK 3D/2 crosspieces Sulfinert®
177 (Gerstel). The OPD transfer line and mixing chamber were heated to 250 °C. The
178 injector and detector temperature were both at 250 °C. The quadrupole, source, and
179 transfer line temperatures were maintained at 150°, 230°, and 280 °C, respectively.

180 A panel of three trained panellists performed a total of six sniffings per sample. These
181 panellists had to smell the effluent of the column and to give a verbal description of
182 each perceived odour and assign it an intensity level, namely 1, 2 or 3. The odour

345

zones reported by each panellist were compared for each retention time and the descriptors were selected according to their frequency of citations. The results were expressed as the ‘‘modified frequency’’ (MF), which was calculated by using the formula $MF(\%) = [F(\%) \times I(\%)]^{1/2}$ proposed by Dravnieks, (1985), in which F is frequency of occurrence and I intensity (Dravnieks, 1985).

2.4. Quantification and identification of aroma compounds by GC-MS analysis

To quantify and identify the volatile compounds of our interest, GC-MS analysis was applied. Two different extraction methods were used, due to the fact that only one was inadequate for determining all of the compounds: headspace sorptive extraction (HSSE) and liquid-liquid extraction (LLE).

In the first extraction method, a total of 43 compounds were determined following the method validated by Callejón, et al., 2008, and used in a previously-published work (Ríos-Reina, Morales, García-González, Amigo, & Callejón, 2018). However, for the special case of sotolon, pantolactone and acetovanillone (polar compounds), the HSSE method was not suitable because of the apolar nature of the polydimethylsiloxane (PDMS) sorbent. Therefore, an LLE method was employed, following the same extraction and analysis methods described above in Section 2.3.4. The samples were analysed in triplicate, and blank runs were undertaken before and after each analysis.

Quantification was performed employing the relative peak area to the internal standard of the target ion of each compound. Calibration curves for each compound were produced by plotting concentrations versus their relative areas.

Odorant identification was performed by mass spectrum matching in the standard NIST 98 library, linear retention indexes (LRIs) on two columns with different polarities (CPWax and HP5), and odour description with experimental and literature data as well as data from the online Flavornet[1] and Pherobase[2] databases. Some compounds were

[1]www.flavornet.org

considered as tentatively identified (TI) because only the odour description and LRIs matched with the literature and databases. LRIs were calculated with the retention times of n-alkanes (C10–C32) by linear interpolation, according to the literature (Antonio César Silva Ferreira, Hogg, & Guedes De Pinho, 2003).

2.5. Statistical analysis

All statistical analyses were performed by using Matlab version 2016a (The Mathworks, Natick, MA) and PLS-toolbox version 7.0.2 (Eigenvector Research Inc., Manson, WA). Principal component analysis (PCA) was performed as an unsupervised method in order to ascertain the degree of differentiation between samples considering the odour zones with MF>80%, identified as variables, autoscaled prior the analysis. Analysis of variance (ANOVA) test of QDA scores of each sample was performed.

3. Results and discussion

3.1. Odour-active profile of the PDO wine vinegars

Among all the odour zones detected in this study (i.e. 225), following the criteria of other authors (Márquez et al., 2013; Vera, Uliaque, Canellas, Escudero, & Nerín, 2012), we considered odour-active those compounds that were detected in at least half of the total sniffing analysis and reached a modified frequency value (MF) higher than 58% (i.e. 3 sniffing with intensity of 2). Therefore, a total of 103 odour active compounds were considered in this study, their corresponding identifications being listed in **Table 1.** 69 of them, moreover, reached an MF higher than 80, being considered as impact odorants.

With regard to the impact odorants for each PDO in the *Reserva* category, the GC-MS-O analyses showed: 44 in vinegars from the PDO *Vinagre de Condado de Huelva* (CRE), 11 reaching the maximum MF (100%); 30 for *Vinagre de Jerez* PDO (JRE), 6 of them obtaining the maximum MF; and *Vinagre de Montilla-Moriles* PDO (MRE) with a

[2] www.pherobase.com

lower number of impact odorant zones up to 25, 6 of them showing 100% MF. The *Pedro Ximénez* category also showed a great aromatic complexity, as reflected in the high number of impact odorants, especially in the case of *Vinagre de Montilla-Moriles* PDO (i.e. 32 and 41 impact odorants in JPX and MPX samples, respectively). In addition, 13 of the impact odorants in JPX reached 100% of the modified frequency, while in the case of MPX these compounds numbered 14.

Independent of category or PDO, the most important aroma compounds in all of the vinegars (MF=100%) were the odour zone identified as butyric acid (LRI 1632) and the odour zone identified as isovaleric acid (LRI 1670). These compounds were both associated with cheesy odour. Different authors (Aceña et al., 2011; Callejón, Morales, Silva Ferreira, et al., 2008; Callejón, Morales, Troncoso, et al., 2008; Charles et al., 2000) have previously described them as an impact odorant in wine vinegars. The large presence of acids in all the wine vinegars was explained by the oxidation of precursor alcohols by the acetic acid bacteria (Charles et al., 2000).

Sotolon (LRI 2221-2238) was another odour compound perceived in all the vinegars with very high MF values (>90%) and with a liquorice odour description. Hence, this compound could be considered as an impact odorant in the Spanish PDO wine vinegars. According to Alexandre et al., 2013, sotolon is formed during the biological aging of the wines used to produce the vinegars (Alexandre, 2013). Thus, sotolon was previously identified as an impact odorant in Sherry vinegars and wines (Callejón, Morales, Silva Ferreira, et al., 2008; Callejón, Morales, Troncoso, et al., 2008; Moreno, Zea, Moyano, & Medina, 2005), being quantified in most aged Sherry vinegar categories (*Reserva* and *Gran Reserva*) and undetected in younger vinegars (Callejón, Morales, Silva Ferreira, et al., 2008). Moreover, it has been described as an impact odorant in sweet wines such as Port and Pedro Ximénez wines (Campo, Cacho, & Ferreira, 2008; A.C. Silva Ferreira, Barbe, & Bertrand, 2003). In Port wines, sotolon is formed through the effects of temperature and oxygen (Martins, Monforte, & Silva

Ferreira, 2013; A.C. Silva Ferreira et al., 2003). In the case of the Pedro Ximénez wine vinegars, the presence of this compound could be due to the effect of temperature on the raw material (sun-dried grape process). In agreement with this, the high MF sotolon for JPX and MPXshould also be highlighted, this being the first time it has been determined as an impact odorant in these sweet wine vinegars.

The other odour zones perceived with a very high MF (>80%) in all of the vinegars were identified as ethyl propionate (LRI 961), furfural & methional (LRI 1454), isobutyric acid (LRI 1565), cis-β-methyl-γ-octalactone (LRI 1968), 4-ethylguaiacol (LRI 2032) and, naturally, acetic acid (LRI 1408) (**Table 1**). The majority have previously been considered as relevant compounds in the final sensory profile of wines and high-quality vinegars aged in wooden barrels (Raquel M. Callejón et al., 2008; Raquel M Callejón et al., 2008; Torrens, Rlu-Aumatell, Vichi, López-Tamames, & Buxaderas, 2010).

In the *Reserva* samples (**Table 1**), ethyl acetate (LRI 948), diacetyl (LRI 976), 2-methy-1-butanol (LRI 1237), β-damascenone (LRI 1820), pantolactone (LRI 2040) and pehnylacetic acid (LRI 2587), were shown to be impact odorants in the CRE and MRE samples, yet not in JRE; this latter presented MF<75% for those compounds. Previous studies of the aromatic profile of vinegars showed that ethyl acetate was one of the volatile compounds with a great influence on the final sensory profile of wine vinegars. In the present study, this compound showed a difference in the MF between the samples of the three PDOs, being higher for MRE and CRE samples.

Similarly, propanoic acid (LRI 1539), benzyl acetate & alfa-terpineol (LRI 1718+1721), ethyl salicylate (LRI 1780), hexanoic acid (LRI 1855), 4-ethylphenol (LRI 2193) and acetovanillone (LRI 2663) appeared to be impact odorants in the CRE and JRE samples, with an MF between 83% and 100%, yet not in MRE (MF<75%). In fact, ethyl salicylate (LRI 1780), an ester formed by the condensation of salicylic acid and ethanol with an unpleasant odour, was not even detected in MRE.

Methionol (LRI 1729) and abhexone (TI-LRI 2255) were impact odorants for JRE and MRE, but not for CRE. The first-mentioned compound, methionol, is mainly produced during alcoholic fermentation by yeasts, *via* amino acid metabolism and was described in the literature as an aroma compound marker in Sherry-type wines subjected to aging (Moreno et al., 2005). Abhexone (TI-LRI 2255) has been identified as one of the impact odorants responsible for the sweet-caramel note in some other food matrices such as coffee and strawberry vinegars (Ubeda et al., 2016; Zellner et al., 2008).

On the other hand, some odorants reached high MF values (MF>80%) in only one of the PDOs. Hence, 7 odour zones identified as ethanol (LRI 953), acetaldehyde diethyl acetal (LRI 969), isobutyl acetate (LRI 1008), 3-methyl-1-butanol (LRI 1256), cis-3-hexenol (LRI 1399), maltol (LRI 1997) and one unknown (LRI 2158) were found as impact odorants only in the *Reserva* sample of *Vinagre de Condado de Huelva* PDO (CRE). These results were expected due to the results observed in previous studies (Amigo, Savorani, Cocchi, Callejón, & Ríos-Reina, 2019; Ríos-Reina, García-González, Callejón, & Amigo, 2018), in which the wine vinegars protected under this PDO also showed a higher presence of ethanol, acetic acid, and some alcohols and esters than the other two PDOs. In the case of JRE, cis-2-nonenal (LRI 1497) and cis-3-hexenyl acetate (TI) with LRI 1336, both with a grassy-vegetal aroma description, together with phenethyl acetate (LRI 1812), with floral nuances were three characteristic impact odorants in JRE. Moreover, it should also be noted that despite the odour zones identified as furfural & methional (LRI 1454) and isobutyric acid (LRI 1565), being impact odorants in all of the samples, they presented the highest MF (MF=100%) for both categories (JRE and JPX) of *Vinagre de Jerez* PDO.

With regard to the possible characteristic impact odorants for MRE category, the odour zone described with empyreumatic odour (LRI 1612 not identified) and the odour zone identified as acetoin (LRI 1323) showed higher MF for this category than for the rest of samples. The important presence of acetoin in *Vinagre de Montilla-Moriles* PDO

samples could be explained by the fact that this compound has been previously described as the odorant showing the highest content in musts from cv. *Pedro Ximénez* grapes - the variety of grape most widely-used in this PDO. This fact also could also explain its high detection frequency in *Pedro Ximénez* samples, whose production uses this grape variety's must or wine. These results were in agreement with those obtained in a previous work, in which acetoin was selected as a marker of this PDO (Ríos-Reina, Segura-Borrego, García-González, Morales, & Callejón, 2019).

On the other hand, in the *Pedro Ximénez* category, some impact odorants appeared to be preserved from the raw characteristics of the PDO wine to which they belong. Some others appeared to be more closely related to these sweet vinegars' specific production processes. Thus, furfural & methional (LRI 1454) and isobutyric acid (LRI 1565) showed an MF of 100% for the *Vinagre de Jerez* PDO independently of the category, as also occurs with phenylacetic acid (LRI 2587) in *Vinagre de Montilla-Moriles* samples. However, the odour zones identified as diethyl malate (LRI 2069), together with cinnamyl alcohol (TI-LRI 2286), with sweet, empyreumatic and spicy odour descriptors, respectively, were the impact odorants for *Pedro Ximénez* samples and not for the *Reserva* category. Some of them have been reported in white dehydrated grapes, or in raisined grapes used to produce sweet wines (Vincenzi et al., 2011), and therefore, the specific production of the sweet wine vinegars could explain their higher presence. Therefore, to produce these sweet vinegars, *Pedro Ximénez* wines or the must of sun-dried *Pedro Ximénez* variety grapes are added during the production process or the maturing process, in the case of JPX and MPX, respectively (BOJA, 03/10/08; BOJA, 16/09/08) .

Moreover, it should be highlighted that the odour zone described as ethanol was not detected in the *Pedro Ximénez* samples, in spite its being an impact odorant in the *Reserva* category. The same occurs with ethanol, ethyl 3-methylpentanoate (LRI 1167), ethyl 4-oxopentanoate (LRI 1616) and ethyl salicylate (LRI 1780).

340 In order to help the study of the aromatic profile of each sample, the total odour zones
341 included in **Table 1** were grouped into 9 categories based on their aroma
342 characteristics: 6 butter-lactic-cheesy, 11 chemicals, 10 empyreumatic, 15 grassy-
343 vegetal, 11 spicy, 15 sweets, 7 florals, 18 fruity and 10 miscellaneous. This last group
344 included odour zones that were described as tempera, sweat, plastic or metallic. **Fig. 1**
345 shows the contribution of each aroma category as a percentage of number of odour
346 zones that were impact odorants in at least one sample with an MF>80% in the bar
347 graph.

348 The 'spicy' group included odour descriptors such as river water, cloves, cinnamon,
349 anise and liquorice. The last descriptor was mainly associated with sotolon (LRI 2221-
350 2238),. As observed in **Fig.1**, this aromatic group presents almost similar percentages
351 in all samples, except for *Pedro Ximénez* that was slightly higher, especially in the case
352 of JPX (around 19%). In the literature these compounds have also showed a
353 contribution to the raisin notes of *Pedro Ximénez* wines (Bakker & Clarke, 2012).

354 The odour zones described as synthetic, rancid or pungent were included in the
355 'chemical' group. The sample that presented the higher percentage for this aromatic
356 group was CRE. In fact, **as Fig. 1** shows, the impact odorants that mainly described
357 this sample are those with a chemical odour (20.5%).

358 'Grassy and vegetable' was the other important odour group (ranging from 23.3 to
359 12.0%). JRE sample presented the highest percentage of impact odorants with 'grassy'
360 aromatic character (Fig.1), with cis-2-nonenal (LRI 1497) and cis-3-hexenyl acetate (TI-
361 LRI 1336) as impact odorants for this sample, in addition to the compounds mentioned
362 above.

363 Within the 'buttery-lactic-cheesy' group according to the results showed in **Fig.1**, it
364 should be highlighted that MRE showed the highest percentage for this aromatic group,
365 with 20.0% of the total, being the main aromatic group for this sample. This was

366 explained mainly by the importance, discussed above, of acetoin in this sample, which
367 has a buttery descriptor (**Table 1**).

368 The 'sweet' group includes odour zones described as caramel, vanilla and cotton
369 candy. In the present study, despite some impact odorants of this group being present
370 in all the samples, the sweet category *Pedro Ximénez* had, as expected, a higher
371 presence of this aromatic character than the '*Reserva*' category (15.6% and 17.1% of
372 the total for JPX and MPX, respectively) because of the specific characteristics of their
373 production. In addition, this aromatic character was one of the main differences
374 observed for the *Pedro Ximénez* samples between the two PDOs, together with the
375 'empyreumatic' character, being higher in MPX than in JPX, whereas the 'spicy'
376 character was greater in JPX. Again, these results could be related to the characteristic
377 wine vinegar-making procedures of these *Pedro Ximénez* vinegars.

378 The 'floral' group includes descriptors such as rose, related mainly to phenylacetic acid
379 (LRI 2587). For this group, although all the samples showed similar percentages, JRE,
380 with 13.3%, is worth noting.

381 The 'empyreumatic' group named is formed by odour zones related to toasted or burnt
382 aroma. It should be highlighted that many of the compounds included in this group
383 were not detected in many of the samples. The vinegar that accounted for the highest
384 percentage of the 'empyreumatic' group was MPX. This sample, in contrast to the JPX
385 sample, is the only one that allows the addition must of sun-dried grapes of the *Pedro*
386 *Ximénez* during the maturing process. Therefore, this could explain that the higher
387 amount of toasted odour characters in this sample. Thus, 2,3-butanedioldiacetate (LRI
388 1529), 2,6-dimethylpyrazine (LRI 1313) and dihydromaltol (LRI 1866) were only
389 detected as impact odorants in the MPX sample, and were not even detected for
390 *Reserva* samples (**Table 1**).

391 The ‘fruity’ and ‘miscellaneous’ aromatic groups were those with the lowest percentage
392 of odour zones. Regarding the first group, some of the aromatic descriptors included as
393 ‘fruity’ were strawberry, banana, apple, cherry and blackberry, their related odorant
394 showing a high MF mainly in the CRE sample (i.e. ethyl isovalerate). This leads to the
395 results shown in **Fig.1**, as CRE was the sample that could have the greatest fruity
396 odour notes due to it presenting the highest percentage of these impact odorants
397 among all the samples studied (9.1%). CRE also showed the highest percentage of
398 ‘miscellaneous’ with regard to the other samples, although it was very low (i.e. 6.8%).
399 This group contains descriptors that, aromatically, were very different, such as
400 tempera, metallic, plastic or sweat, many of them categorized as unpleasant. Following
401 the criteria of other authors (Ubeda et al., 2016), they could not be classified in the
402 other abovementioned groups.

403 The results presented in **Fig.1**, clearly showed the main differences in the aromatic
404 profile between the PDOs and the categories:Regarding *Vinagre de Jerez* PDO, JRE
405 had a remarkable ‘grassy-vegetal’ aromatic characteristic, followed by ‘spicy’, ‘sweet’
406 and ‘floral’ nuances. The sweet category JPX was similar to JRE, but with a higher
407 presence of ‘spicy’ and ‘sweet’ aromatic characters. *Vinagre de Montilla-Moriles*
408 samples have an important ‘buttery-lactic’ odour, with some ‘chemical’ nuances for
409 MRE and ‘empyreumatic’, ‘spicy’ and ‘sweet’ nuances for the MPX category. Moreover,
410 the main difference between the *Pedro Ximénez* samples from each PDO was, apart
411 from the higher amount of ‘sweet’ impact odorants in MPX than in JPX, that JPX
412 presented a higher percentage of ‘floral’ impact odorants (12.5%) than MPX (9.8%), the
413 same occurs with JRE and MRE, while MPX presented a higher percentage of
414 ‘empyreumatic’ odour zones (12.2%) than JPX (6.3%) (**Fig.1**). Finally, *Vinagre de*
415 *Condado de Huelva* PDO showed a strong presence of ‘chemical’ odorants, followed
416 by ‘spicy’ and some more ‘fruity’ aromatic characters than the others.

417 **3.2. Principal component analysis**

418 The results discussed in the previous section showed the presence of differences
419 between PDOs and categories. This could be useful for authenticating each one, as
420 well as for discriminating between them. Hence, principal component analysis (PCA)
421 was performed by using only the impact odorants with MF>80% at least for a sample in
422 order to verify if it could be possible to clearly differentiate the 3 PDO wine vinegars
423 and their categories in terms of their aromatic profile. This multivariate analysis enabled
424 a relationship between the different odorant compounds and the wine vinegars to be
425 established by finding the odorants responsible for the variability.

426 First, a PCA model was built for all of the samples in order to study the differences
427 between categories (**Fig 2.A**). The first two components explained 59.12% of total
428 variance. As shown in the scores plot of **Fig 2.A**, the first principal component (PC1)
429 separates the *Reserva* vinegars from the *Pedro Ximénez* vinegars. According to the
430 loadings, and in accordance with the results discussed above, *Pedro Ximénez* vinegars
431 were more correlated with 'sweet' odorants, such as vanillin (LRI 2595) and p-
432 vinylguaiacol (2203), as well as with all the 'empyreumatic' or toasted odours identified
433 as 2,3-butanedioldiacetate. Moreover, some differences were observed between the
434 two *Pedro Ximénez* samples. Thus, JPX appeared to show a higher relationship to
435 sotolon (LRI 2221-2238) with spicy odour, whereas, MPX showed a higher correlation
436 with 3-nonen-2-one (LRI 1509) of the 'spicy' group, and 2,3-butanedioldiacetate (LRI
437 1529) from the 'empyreumatic' group.

438 *Reserva* vinegars were correlated more with compounds having 'chemical' and 'grassy'
439 nuances (i.e. ethanol (LRI 953), propanoic acid (LRI 1539), ethyl propionate (LRI 961),
440 methionol (LRI 1729) and *cis*-2-nonenal (LRI 1497)). In addition, the relationship of this
441 category with more 'spicy' odorants, such as cis-β-methyl-γ-octalactone (LRI 1968)
442 could be observed, as well as 'fruity' odorants such as ethyl octanoate (LRI 1440) and
443 ethyl isovalerate (LRI 1059).

444 In order to better assess the differences between PDOs, once all of the samples were
445 studied together, a new PCA model was built including only the *Reserva* samples (Fig.
446 2B). As could be seen, each PDO was separated into its own specific quadrant on the
447 scores plot. Thus, CRE was placed in the extremely positive side of PC1, MRE was
448 placed on the negative side of PC1 and PC2, while JRE was placed on the negative
449 side of PC1 and on the most positive side of PC2. Hence, PC1 differentiated CRE from
450 the others, and PC2 differentiated JRE from MRE.

451 According to the loadings, three of the five 'grassy-vegetable' odorants, furfural&
452 methional (LRI 1545), cis-3-hexenyl acetate (LRI1315) and cis-nonenal (LRI 1497)
453 showed a high correlation with JRE, as they were placed on the negative side of PC1
454 and positive side of PC2. The other remaining two 'grassy' odorantswere placed on the
455 positive side of PC1 and hence were more related to CRE. Moreover, the 'chemical'
456 odorants most closely related to JRE were ethyl propionate (LRI 961), and propanoic
457 acid (LRI 1539) whereas the 'chemical' odorants most closely related to CRE were
458 isobutyl acetate (LRI 1008), ethanol (LRI 953) and ethyl acetate (LRI 948), among
459 others. In MRE a close relationship can be observed with acetoin, with a 'buttery-lactic'
460 odour, as well as with abhexone (TI) with 'sweet' odour, the compound grouped as
461 'miscellaneous' (LRI 3082), which has an odorant descriptor of tempera, and the
462 unknown compounds LRI 1740 and 1612, with bitumen and toasted odours.

463 **3.3. Odour activity values**

464 Once the contribution of each volatile compound to the aroma of the wine vinegars
465 had been evaluated by GC-MS-O, a quantitative evaluation of these aromas was
466 performed by the mean of their odour activity values (OAV). Using both
467 evaluations, the key odorants in each PDO and category could be correctly
468 ascertained. OAV is obtained by dividing the concentration of the compound by its
469 recognition threshold in a suitable matrix. It is, therefore, linearly proportional to
470 concentration and threshold (Brattoli et al., 2013; Callejón, Morales, Silva Ferreira, et
al., 2008). This means that when a single

compound exceeds its odour threshold, it should strongly contribute to the overall aroma (Delahunty et al., 2006). Therefore, it is generally assumed that the odorants with higher OAVs (OAV >1) contribute more strongly to the overall aroma.

The odour thresholds were, therefore, calculated for the odorants that reached the highest modified frequencies (MF>80%) in GC-MS-O analysis or that were detected by all the panellists in the total of sniffing, and with an available standard. As a result, those compounds that were only tentatively identified were not considered. The OAVs were calculated for 40 odorants, and those achieving an OAV >1 are shown in **Table 2**. Some of these odour thresholds were calculated and reported in a previous work (Callejón, Morales, Silva Ferreira, et al., 2008) while in this study 6 odour thresholds (acetovanillone, guaiacol, propanoic acid, butyric acid and cis-3-hexenol) were determined in vinegars for the first time.

As can be seen in **Table 2**, the odour threshold ranges were from 0.04 µg/L for diacetyl to 133 mg/L for acetaldehyde diethyl acetal. Compounds in the table are ranked according to the maximum OAV reached in the five PDO wine vinegars under study by GC-MS-O. It can be seen that nearly all of the compounds with high MF values also had high OAVs, confirming the GC-MS-O results discussed in sections 3.1. and 3.2.

The first maximum OAV in rank was obtained for diacetyl (LRI 976), specifically in the MRE sample, followed by its sweet category MPX vinegar. On the other hand, other compounds that also presented the maximum OAV for this PDO (MRE and MPX) was acetoin (LRI 1323) and vanillin (LRI 2595), providing buttery-lactic-cheesy and sweet odours (**Table 1 and 2**).

The second maximum OAV was found for acetaldehyde diethyl acetal (LRI 969), specifically for CRE wine vinegar. A great difference between this OAV and the one obtained for the other samples can be observed, the lowest OAV being the one obtained for the MRE sample. This fact, together with the results shown in Fig. 1, could

497 confirm the high contribution of the chemical character of this PDO. Moreover, 4-
498 ethylphenol (LRI 2193) showed high OAVs for all the samples, independently of the
499 category or PDO due to it presenting a low odour threshold. The other compounds that
500 presented OAV>100 for almost all the samples were sotolon, isovaleric acid, ethyl
501 isovalerate and acetoin.

502 The joint results OAV and GC-MS-O enabled the characteristic key odorants for each
503 PDO and category to be selected. This is because OAV is useful for complementing
504 and improving the suitability of the GC-MS-O results. These compounds were those
505 that in general fulfil the conditions of having concentrations above their odour
506 thresholds and an MF> 80%.

507 Thus, the compounds that presented high MF and OAV>1 for JRE, and therefore could
508 be selected as the characteristic odour active compounds for this category, were ethyl
509 propionate (LRI 961), ethyl octanoate (LRI 1440), propanoic acid (LRI 1539), phenetyl
510 acetate (LRI 1812) and 4-ethylphenol (LRI 2193). There were also some other
511 compounds that showed the highest MF and therefore, could be impact odorants for
512 this sample, but their OAVs could not be calculated. These compounds were cis-3-
513 hexenyl acetate (LRI 1336) and cis-2-nonenal (LRI1497), both with grassy aromatic
514 character. In JPX, the odorants with high MF and OAV were diacetyl (LRI 976) and
515 methional & furfural (LRI 1454) together with 6,7-dihydro-7-hydroxylinalool (LRI 1974)
516 whose OAV was not possible to study.

517 According to the same criteria, acetoin (LRI 1323) was the odorant studied that
518 showed a high MF and OAV>1 for MRE sample, providing a strong 'buttery-lactic'
519 aroma. In addition, abhexone (TI-LRI 2255) could also differentiate MRE from the
520 other samples due to its higher MF, although its OAV was not evaluated. More impact
521 odorants could be considered for inclusion in MPX category. Thus, ethyl
522 phenylacetate (LRI1790) and vanillin (LRI 2595) could be selected as characteristic
523 odour active compounds for MPX due to their high MF and OAVs>1, together with
2,6-dimethylpyrazine (LRI 1313),

2,3-butanedioldiacetate (LRI 1529) and dihydromaltol (LRI 1866), all three with an empyreumatic characters, as well as ethyl cyclohexanoate (LRI 1428), 3-nonen-2-one (LRI 1509), β-damascenone (LRI 1820), p-vinylguaiacol (LRI 2203) and benzoic acid (LRI 2513), whose OAVs were not possible to calculate, even though they presented the highest MF values in this sample.

Finally, acetaldehyde diethylacetal (LRI 969) and isobutyl acetate (LRI 1008), with chemical descriptors, and ethyl isovalerate (LRI 1059) and guaiacol (LRI 1860), could be selected as the characteristic odour active compounds for CRE. Therefore, they are useful for discriminating it, due to the fact that they gave their highest MF and OAV>1 in CRE. Moreover, some other compounds had OAV that were not possible to evaluate, but which showed the highest MF for this sample. These were: were ethanol (LRI 953), ethyl acetate (LRI 948) and 3-methyl-1-butanol (LRI 1256), that provide chemical notes, and ethyl salicylate (LRI 1780), β-ionone (LRI 1936) and maltol (LRI 1997).

3.4. Quantitative sensory analysis (QDA) and the relation with GC-MS-O results

The wine vinegar samples analysed by GC-MS-O were also described by an expert sensory panel. Each sample was submitted to QDA using the selected attributes and the results were represented with a spider chart in **Figure 3**. The scores the experts gave to the descriptors ethyl acetate, pungent sensation, woody odour, sweet aroma, vanilla, raisin and bitter almond were significantly different among the samples (according to $p<0.05$ obtained in ANOVA) (**Figure 3**). Among them, ethyl acetate, pungent sensation, vanilla and bitter almond reached the highest values in CRE, sweet aroma in MPX and raisin in JPX.

In order to compare the results from QDA and GC-MS-O analysis, the selected descriptors of the QDA were grouped according to the most appropriate aromatic group of the GC-MS-O analysis (**Table 1**), also in accordance with other authors (W. Tesfaye et al., 2010). Thus, the results of pungent, ethyl acetate and wine descriptors were

matched up with the group of ‘chemicals’, liquorice character with the ‘spicy’ group, acid and red fruits as ‘fruity’, sweet and vanilla descriptors were grouped together as ‘sweet’, woody/toasty odour was related to ‘empyreumatic’ and finally bitter almond and leather-old were included in the miscellaneous group. Moreover, the scale of the sensory test used (i.e. out of 10) was converted to an out of 100 scale, as the results obtained in the GC-MS-O analysis as shown in Fig. 1 (i.e. percentage of number of odor zones with an MF>80%). Comparative spider charts of these data are shown in **Fig. 4.** As can be seen, the QDA and the GC-MS-O results were consistent. Thus, pungent sensation, ethyl acetate and wine character, all classified as ‘chemical’, reached the highest scores for CRE in the QDA and GC-MS-O analysis, followed by the MRE sample, while in both the GC-MS-O and QDA results JPX and MPX reached the highest scores for the ‘spicy’ or licorice character and sweet aromas, respectively. On the other hand, red and citric fruits were, in general, the attributes that accounted for the lowest marks, although CRE showed slightly higher scores than the other samples. The GC-MS-O results also showed greater relationship between this ‘fruity’ character and CRE.

The scores obtained by the two *Pedro Ximénez* samples were similar for all the attributes, except for ‘sweet’, ‘spicy’ and ‘empyreumatic’. The scores given to these descriptors differed between the two PDOs, as seen in the GC-MS-O analysis. The licorice attribute, which was related to ‘spicy’ character, obtained the highest scores for JPX, whereas the descriptors vanilla, raisin and sweet (i.e. sweet aromas) reached the highest values for MPX, once again these results being consistent with the results obtained by GC-MS-O. In addition, MPX showed higher scores for empyreumatic odorants than JPX, as was also the case for their related woody descriptor.

In addition, the ‘buttery-lactic-cheesy’, ‘grassy-vegetal-green’, ‘floral’ and ‘miscellaneous’ groups, previously considered in GC-MS-O analysis, could not be compared as there was no correspondence with the attributes selected in the QDA.

Nonetheless, within them, it should be highlighted that all the panelists marked the perception of the attribute 'cheesy' for the MRE sample, and 'green' for the JRE sample in the list of optional list. These results were consistent with the high relevance of the 'grassy-vegetal-green' aromatic group obtained the GC-MS-O profile of JRE sample, the same occurring with 'buttery-lactic-cheesy' group and MRE wine vinegar. In general, the good relation observed between sensory descriptors and aroma compounds detected by GC-MS-O analysis should be noted, reinforcing the reliability of GC-MS-O.

4. Conclusions

The results showed that some differences were observed regarding the aromatic profile of each PDO and category (*Reserva* and *Pedro Ximénez*) due to the presence of impact odorants that were shown to be characteristic for each kind of wine vinegar. Hence, these compounds could be selected as markers or key odorants useful for their discrimination and authentication.

Vinagre de Jerez PDO could be termed as having a remarkable 'grassy-vegetal' aroma for the *Reserva* category, with some 'floral' nuances according to the high percentage of impact odorants with these nuances. Moreover, 7 impact odorants were selected as characteristic for this categoryaccording to GC-MS-O and OAV results: ethyl propionate, ethyl octanoate, propanoic acid, phenetyl acetate and 4-ethylphenol. Moreover, cis-2-nonenal and cis-3-hexenyl acetate Moreover, its sweet category *Pedro Ximénez* seem to have more 'spicy' and 'sweet' nuances, highlighting diacetyl and methional & furfural as characteristic impact odorants for the JPX vinegar.

The *Reserva* category of *Vinagre de Montilla-Moriles* PDO have an important and characteristic 'buttery-lactic' aroma mainly provided by its impact odorant acetoin according to its OAV and MF, and abhexone according only to its MF. Its sweet *Pedro Ximénez* category, showed more 'empyreumatic', 'spicy' and 'sweet' nuances, and

showed 10 remarkable key odorants: ethyl phenylacetate and vanillin according to both MF and OAVs, together with 2,3-butanedioldiacetate, 2,6-dimethylpyrazine, dihydromaltol, ethyl cyclohexanoate, 3-nonen-2-one, β-damascenone, p-vinylguaiacol and benzoic acid according only to the high MF values.

The *Reserva* sample of *Vinagre de Condado de Huelva* PDO showed a stronger 'chemical' aroma, and presented 8 keyimpact odorants: acetaldehyde diethylacetal and isobutyl acetate according to their OAV and MF, together with ethanol, ethyl acetate, 3-methyl-1-butanol, ethyl salicylate, β-ionone and maltol that were also impact odorants according only to their high MF. This PDO also presented more 'fruity' aromatic characters than the others.

Furthermore, the sweet samples differed from each other due to a higher amount of 'sweet' and 'empyreumatic' impact odorants in the *Pedro Ximénez* category of *Vinagre de Montilla-Moriles* PDO than in *Vinagre de Jerez* PDO.

With regard to the sensory analysis, ethyl acetate, pungent sensation, vanilla and bitter almond attributes reached the highest values in the CRE sample. The sweet attribute was more prevalent in MPX, while JPX obtained the highest raisin attribute scores. Furthermore, a good correlation between sensory descriptors and aroma compounds detected by GC-MS-O analysis was observed, reinforcing GC-MS-O reliability.

The differences observed by the modified frequencies studied by GC-MS-O, the sensory scores and the OAVs enabled PDOs and categories (aged and sweet) to be characterised and differentiated. However, in order to attain a complete analysis of the key odorants and to be able to perform a classificatory approach, further analysis, including more samples and categories should be performed, as should studies of recombination models and omission experiments.

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7. Figure captions

Figure 1. Bar graph of the contribution of each aroma category as a percentage of the number of odour zones that are impact odorants in each sample.

Figure 2. Scores and loadings plots on the planes comprising the first two principal components (PC1 against PC2). PCA model obtained by the compounds with MF>80% of the total of samples (A) and PCA model developed only including "*Reserva*" samples (B). The compound names corresponding to the numbers of variables are located in Table 1.

Figure 3. Spider chart of the sensory profile of mean attribute values for the five samples under study.

Figure 4. Comparative spider charts between the sensory descriptors obtained by QDA and the aroma compounds detected by GC-MS-O analysis for each sample under study.

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TABLES

Table 1. Odour-active compounds in the five wine vinegars detected and described by the sniffing panel.

LRI[a]		Odour descriptor	Aroma category[b]	Odorant	CRE		JRE		MRE		JPX		MPX	
DB-Wax	HP5				F[c]	FM[d]	F	FM	F	FM	F	FM	F	FM
948	624	Glue	Ch	ethyl acetate	6	91	5	75	6	82	6	82	6	71
953		Alcohol	Ch	ethanol	6	82	3	58	5	75	0	0	0	0
961	527	Synthetic, rancid, plastic	Ch	ethyl propionate	5	83	5	91	6	82	6	82	6	82
969		Plastic, synthetic, grass	Ch	acetaldehyde diethylacetal	6	91	3	58	0	0	5	53	6	82
976		Butter	BLC	diacetyl	5	91	3	65	5	91	6	100	6	91
990		Strawberry	Fr	methyl butyrate	4	67	3	71	3	71	2	58	5	75
1008	732	Plastic, medicinal	Ch	isobutyl acetate	6	82	3	58	0	0	5	53	0	0
1030		Strawberry, sweet	Fr	ethyl butyrate	5	75	2	33	4	47	0	0	5	53
1041	806	Acid strawberry, banana	Fr	ethyl 2-methylbutyrate	3	58	5	75	2	33	4	47	5	53
1047		Acid strawberry, fruity	Fr	n-butyl acetate	3	41	3	71	6	58	6	71	6	82
1059		Acid strawberry, banana	Fr	ethyl isovalerate	6	82	2	41	4	47	4	67	0	0
1082		Rubber, sweat	Em	isobutanol	6	58	1	24	0	0	4	47	6	58
1106	851	Banana	Fr	isoamyl acetate	4	75	2	33	3	58	0	0	4	47
1167	853	Medicial, sweet fruit	Fr	ethyl 3-methylpentanoate	3	41	3	58	2	33	0	0	0	0
1212		Sintetic	Ch	unknown	5	53	0	0	5	53	4	67	0	0
1237	721	Green, rancid, alcohol	Ch	2-methyl-1-butanol	6	82	3	58	6	82	2	33	6	82
1256	736	Rancid	Ch	3-methyl-1-butanol	5	83	1	24	3	71	0	0	3	58
1309		Grass, earth, vegetable, plastic	GV	1-octen-3-one	5	53	2	47	0	0	0	0	5	75
1313		Burnt, boiled potato	Em	2,6-dimethylpyrazine	5	53	0	0	0	0	0	0	6	82
1315		Vegetable, gas, rancid	GV	1-octen-3-one	3	58	0	0	5	75	6	71	0	0
1323		Milk, dairy	BLC	acetoin	3	58	4	75	6	82	5	75	5	75
1336		Synthetic, green	GV	cis-3-hexenyl acetate (TI)	3	58	5	91	4	47	3	58	3	41
1345		Fruit, sweet	Fr	ethyl heptanoate (TI)	3	58	4	67	5	65	5	53	4	47
1364	779	Toasted corn, rancid, rubber	Em	3-hydroxy-2-pentanone	6	100	5	75	6	82	6	91	6	100
1385		Geen, humidity, tempera	GV	unknown	4	67	0	0	2	33	1	24	0	0
1399		Grass, green leaf, herb	GV	cis-3-hexenol	6	82	3	58	2	33	5	75	4	47
1408		Pungent, synthetic	Ch	acetic acid	6	100	4	82	4	82	6	100	6	100
1428		Fruit, medicinal, green	GV	ethyl cyclohexanoate	6	58	5	75	6	58	3	41	6	82

1436		Toasted corn, caramel toasted	Em	2-furanmethanethiol	0	0	0	0	0	0	5	53	0	0
1440		Fruit, strawberry	Fr	ethyl octanoate	6	82	6	91	2	47	4	47	5	65
1454	805	Baked potatoes	GV	methional (& furfural)	6	82	6	100	6	91	6	100	6	91
1465		Cherry	Fr	benzaldehyde	2	33	5	75	0	0	4	67	4	67
1497		Cooked vegetables, roasted potatoes	GV	*cis*-2-nonenal	2	33	5	91	0	0	0	0	3	58
1509		River water	Sp	3-nonen-2-one	6	82	4	67	5	75	6	82	6	100
1529	957	Toasted	Em	2,3-butanedioldiacetate (TI)	0	0	0	0	0	0	4	67	6	100
1535		Sweet	Sw	ethyl nonanoate	6	82	4	47	6	82	6	82	6	82
1539	730	Fat, rancid, plastic	Ch	propanoic acid	6	82	6	91	4	67	3	65	2	47
1565		Cheese, vomit	BLC	isobutyric acid	6	82	6	100	6	82	6	100	6	91
1583	789	Fruit, sweety	Fr	2,3-butanediol (TI)	4	58	3	41	2	33	5	53	2	33
1612		Toasted caramel	Em	unknown	4	58	0	0	6	91	6	82	5	53
1616	1140	Fruit	Fr	pentanoic acid, 4-oxo-, ethyl ester	1	33	4	67	0	0	0	0	0	0
1629		Roasted corn, toasted cereal	Em	2-acetylpyrazine	4	67	5	75	5	75	4	67	4	67
1632	873	Cheese, vomit	BLC	butyric acid	6	100	6	100	6	100	6	100	6	100
1644		Fruit, honey	Fr	ethyl benzoate	5	75	3	50	5	53	5	53	6	82
1664		Burned, toasted, earth, coffe	Em	furfuryl alcohol	2	33	4	67	0	0	0	0	1	24
1670	972	Cheese	BLC	isovaleric acid	6	100	6	100	6	100	6	100	6	100
1690	1194	Sweet, quince, fruit compote	Fr	diethyl succinate	3	58	3	41	3	41	3	41	3	41
1718&1721		Mint, grass, herbaceous, wet land	GV	benzyl acetate (&alfa-terpineol)	6	100	6	82	4	67	6	100	6	91
1729		Earth, potato, green	GV	methionol	5	75	6	82	6	82	5	75	5	75
1732		River water, plastic	Mi	unknown	5	75	2	53	4	67	2	47	6	91
1736		Green, roasted apple	GV	unknown	4	67	2	58	2	47	2	47	3	41
1740		Plastic, bitumen, sweat, methane gas	Ch	unknown	2	47	3	58	5	75	5	83	6	82
1780		Sweat, urine	Mi	ethyl salicylate	5	91	5	83	0	0	0	0	0	0
1790		Floral, rose	Fl	ethyl phenylacetate	6	82	6	82	5	75	6	82	6	100
1812		Floral, honey, rose	Fl	phenetyl acetate	2	47	5	83	5	75	2	47	4	67
1820		Apple compote, quince	Fr	β-damascenone	5	91	4	67	6	82	5	83	6	100
1855	1050	Oil, baked potatoe, chickpea	Gv	hexanoic acid	6	100	5	83	3	65	6	100	6	82
1860		River water, salty	Sp	guaiacol	6	100	4	82	4	82	2	58	6	82
1866		Toasted caramel	Em	dihydromaltol	0	0	0	0	0	0	4	67	4	82
1899	1055	Cherry, fruit, rose	Fr	benzyl alcohol	5	53	2	58	3	58	4	67	5	75
1919	1135	Rose, talcum powder	Fl	phenethyl alcohol	6	100	6	91	6	100	6	100	5	75
1936		Floral	Fl	β-ionone	6	82	4	75	5	53	3	41	5	75
1957		River water, clove	Sp	4-methylguaiacol	4	58	2	33	4	47	5	53	5	75
1968		Coconut, ripe	Sp	cis-β-methyl-γ-octalactone	6	100	6	100	6	100	6	82	6	82
1974		River water, grass	GV	6,7-dihydro-7-hydroxylinalool	4	67	4	67	1	41	6	82	2	47
1997		Sweet, toasted caramel,raisins	Sw	maltol	6	82	3	58	4	47	4	67	5	65
2009		Metallic, river water, toasted	Mi	unknown	0	0	0	0	4	67	5	53	5	53

2032		Cinnamon clove, hazelnut	Sp	4-ethylguaiacol	6	82	6	82	6	82	6	82	6	82
2040		Quince, fruit, sweet	Fr	pantolactone	6	82	5	83	5	75	4	67	6	71
2069		Coconut, brown sugar	Sw	diethyl malate	3	41	4	58	5	53	6	82	6	82
2077		Tempera, powder, toasted corn	Mi	p-cresol	3	58	4	67	3	41	4	47	4	67
2090		Fat, grass, manure	Mi	octanoic Acid	6	82	4	67	6	82	6	82	6	82
2105		Cotton candy, vanilla, coconut	Sw	homofuraneol	3	58	5	65	4	47	3	41	3	41
2130		Sweet camphor, plastic	Sw	ethyl cinnamate (TI)	3	41	5	75	3	41	3	58	0	0
2158		Ripe fruit, clove, aniseed	Sp	unknown	6	91	3	58	3	58	4	47	5	53
2171		Clove, cinnamon	Sp	eugenol	3	58	4	67	4	67	5	75	4	58
2184		Burned, synthetic, betun	Em	unknown	6	91	5	83	5	75	5	75	6	91
2193		Tempera, leather	Mi	4-ethylphenol	6	82	5	91	1	41	2	53	0	0
2203	1327	River warer, vanille, coconut	Sw	p-vinylguaiacol	6	82	6	82	5	75	6	82	6	100
2221-2238		Licorice	Sp	sotolon	5	91	6	100	6	100	6	100	6	100
2255		Sweet, candy	Sw	abhexone (TI)	4	67	6	82	5	91	4	75	6	82
2286		Anise, clove, river water	Sp	cinnamyl alcohol (TI)	6	82	5	75	4	75	6	100	6	100
2327		Anise, toothpaste	Sp	unknown	6	82	6	82	4	67	6	100	6	100
2344-2355		Mushroom, vegetal, metallic	GV	unknown	6	91	6	91	6	82	5	75	6	91
2378		Dairy, floral, honey, body milk	BLC	γ-dodecalactone	4	67	3	58	4	47	4	47	3	41
2415		Unpleasant	Mi	unknown	0	0	3	58	0	0	5	75	0	0
2429		Floral	Fl	unknown	5	75	4	67	4	58	0	0	3	41
2455		Shyntetic, river water	Mi	unknown	0	0	3	58	2	33	6	58	5	53
2481		Cinnamon, spicy	Sp	unknown	6	58	4	47	0	0	6	82	5	65
2513		Floral	Fl	benzoic acid (TI)	4	67	2	33	5	65	1	24	6	82
2524		Mushroom, baked potatoe	GV	unknown	4	58	0	0	0	0	4	47	3	41
2537		Vanille, canndy	Sw	5-hydroxymethyl-2-furaldehyde	0	0	4	67	5	75	4	67	4	47
2587		Rose	Fl	phenylacetic acid	6	100	4	67	6	100	4	82	6	100
2595	1407	Vanille, sugar	Sw	vanillin	3	71	5	91	3	71	3	71	6	100
2614		Vanille, honey	Sw	methyl vanillate	4	67	5	75	3	58	3	58	4	58
2646		Fish, metallic, sweat	Mi	unknown	5	53	4	67	3	58	5	75	5	53
2647		Sweet colony, vanille	Sw	unknown	0	0	0	0	0	0	4	67	5	75
2663		Honey, quince	Sw	acetovanillone	6	100	6	91	5	75	6	100	5	53
2758		Vanille	Sw	vanillyl alcohol (TI)	3	41	4	47	4	47	4	67	4	47
2968		Blackberry, violet	Sw	unknown	3	71	4	67	4	67	6	82	6	82
2985		Floral, sweet, honey	Sw	unknown	6	82	5	75	6	82	5	75	6	91
3043		Blackberry	Fr	unknown	6	71	2	47	4	67	5	75	4	67
3082	1469	Tempera	Mi	unknown	4	67	4	67	4	75	5	91	4	67

[a] LRI: Linear Retention Index by CP-WAX and HP-5 columns

[b] Aroma category: Ch “chemical”; BLC “butter-lactic-cheesy”; Fr “fruity”; Em “empyreumatic”; GV “grassy-vegetal”; Sp “spicy”; Sw “sweet”; Mi “miscellaneous”; Fl “floral”.

[c] F: frequency of occurrence (number of times that odour zone is perceived by the panel).

[d] MF: modified frequency (%).

Table 2. Odour-Activity Values (OAV), Odour thresholds and concentration of impact odorants.

Odorant	Odour threshold (µg/L)	OAV max	OAV					Aroma category[c]
			CRE	JRE	MRE	JPX	MPX	
diacetyl[a]	0.04	11255	2588	3617	11255	3477	8968	BLC
acetaldehyde diethylacetal[a]	133000	3131	3131	910	6.0	527	122	Ch
4-ethylphenol[a]	4	1282	1268	1282	1095	1264	852	Mi
sotolon[a]	16	1092	1092	589		111	302	Sp
isovaleric acid[a]	150	988	11	266	988	325	336	BLC
ethyl isovalerate[a]	4.4	909	909	701	250	727	521	Fr
acetoin[a]	8800	386	50	101	386	128	241	BLC
vanillin[a]	94	175	2.4	64	170	35	175	Sw
phenethyl acetate[a]	88	61	103	76	77	64	75	Fl
phenethyl alcohol[b]	1400	57	57	31	40	27	48	Fl
isobutyric acid[b]	1500	56	30	56	43	31	24	BLC
acetovanillone	324	42	12	42	3.7	10	6	Sw
ethyl phenylacetate[b]	148	29	29	22	22	18	22	Fl
isobutyl acetate[a]	177	19	17	19	14	7.9	11	Ch
ethyl octanoate[a]	62	15	15	3.0		3.3	1.2	Fr
guaiacol	8.38	14	14	7.7	14	8.1	6.0	Sp
furfural[a]	6200	10	2.7	2.3	7.8	10	2.6	GV
propanoic acid	15072	8.5	5.7	8.5	6.5	4.8	5.9	Ch
ethyl propionate[a]	516	5.0	4.7	5.0	1.6	2.1	2.6	Ch
octanoic acid[a]	987	4.2		4.2	0.6	0.1		Mi
2-methyl-1-butanol[b]	12200	2.4	2.1	1.7	2.4	1.0	2.2	Ch
butyric acid	35835	1.8	1.2	1.8	0.8	0.8	0.8	BLC
pantolactone	18600	1.7	0.7	1.7	0.4	0.3	0.5	Fr
4-ethylguaiacol	28	1.2	1.0	1.2	0.8	1.0	1.1	Sp
acetic acid*	700.0	135.7	114.3	135.7	114.3	128.6	85.7	Ch

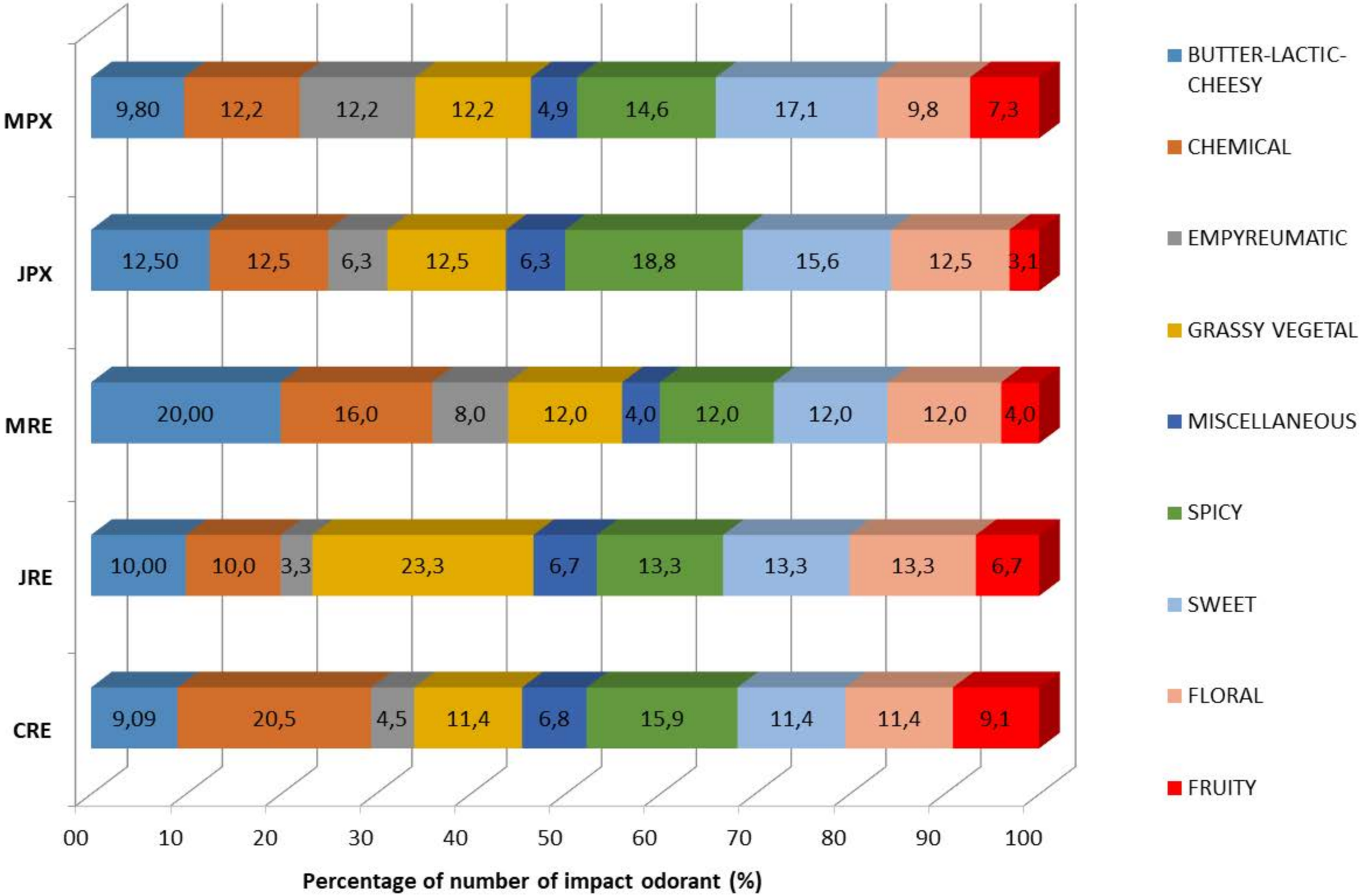
Notes: **acetic acid calculated by their grade acetic. [a]: reported in a previous work (Callejón et al., 2008), [b]: reported by Aceña et al., 2011.[c]Aroma category: Ch "chemical"; BLC "butter-lactic-cheesy"; Fr "fruity"; Em "empyreumatic"; GV "grassy-vegetal"; Sp "spicy"; Sw "sweet"; Mi "miscellaneous"; Fl "floral".*

***Declaration of Interest Statement**

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:


MPX
JPX
MRE
JRE
CRE
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11,4
11,4
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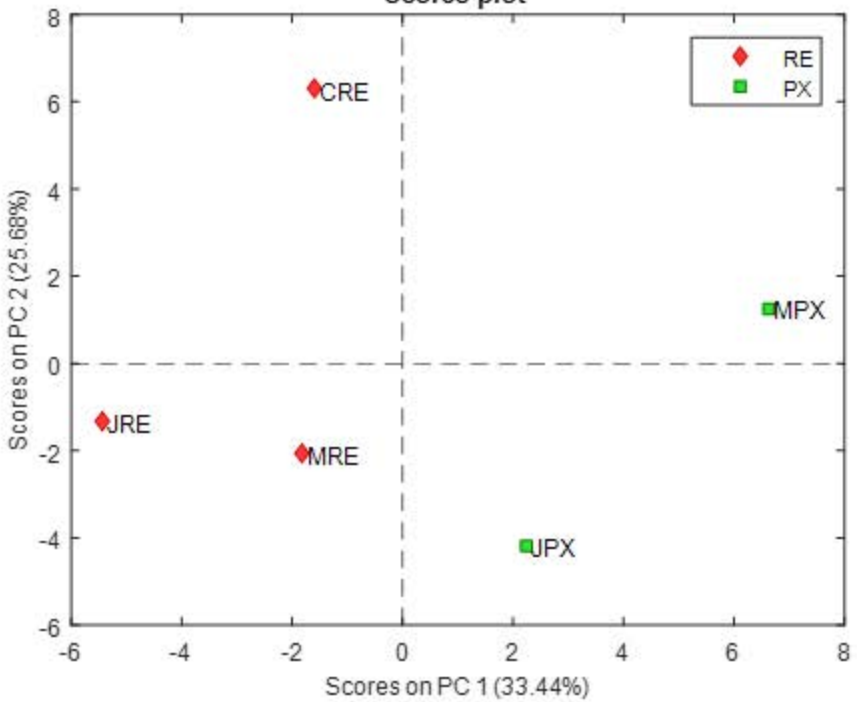
10
20
30
40
50
60
70
80
90
100
Percentage of number of impact odorant (%)
BUTTER-LACTIC-CHEESY
CHEMICAL
EMPYREUMATIC
GRASSY VEGETAL
MISCELLANEOUS
SPICY
SWEET
FLORAL
FRUITY

A)


Scores plot
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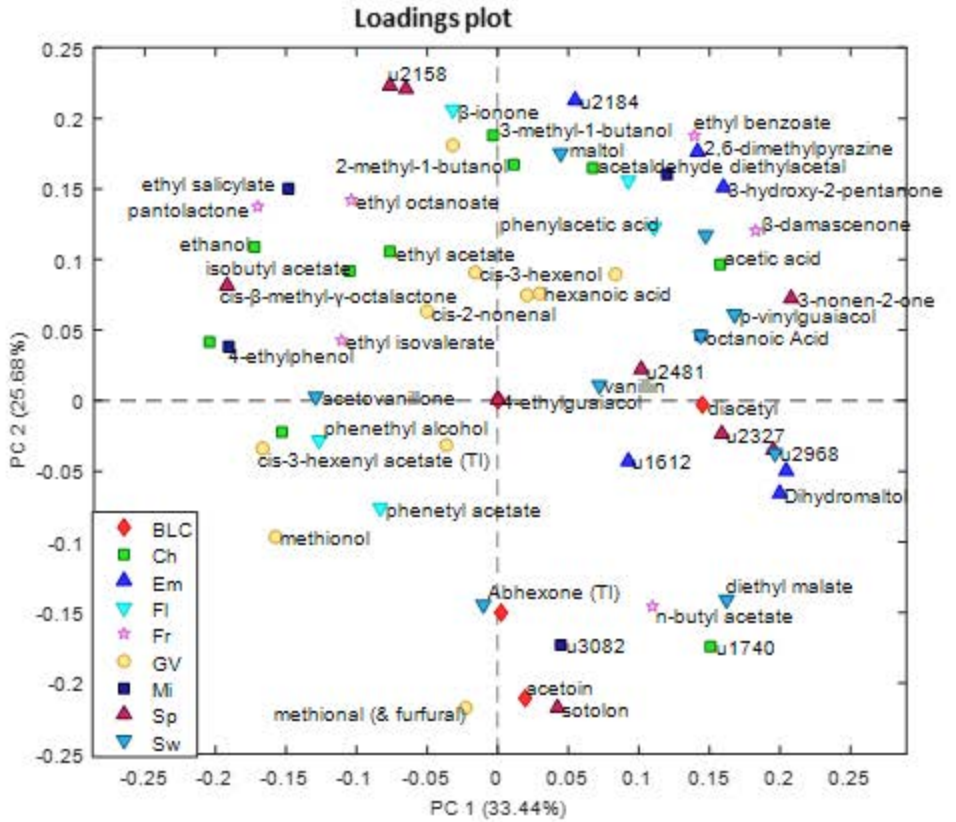
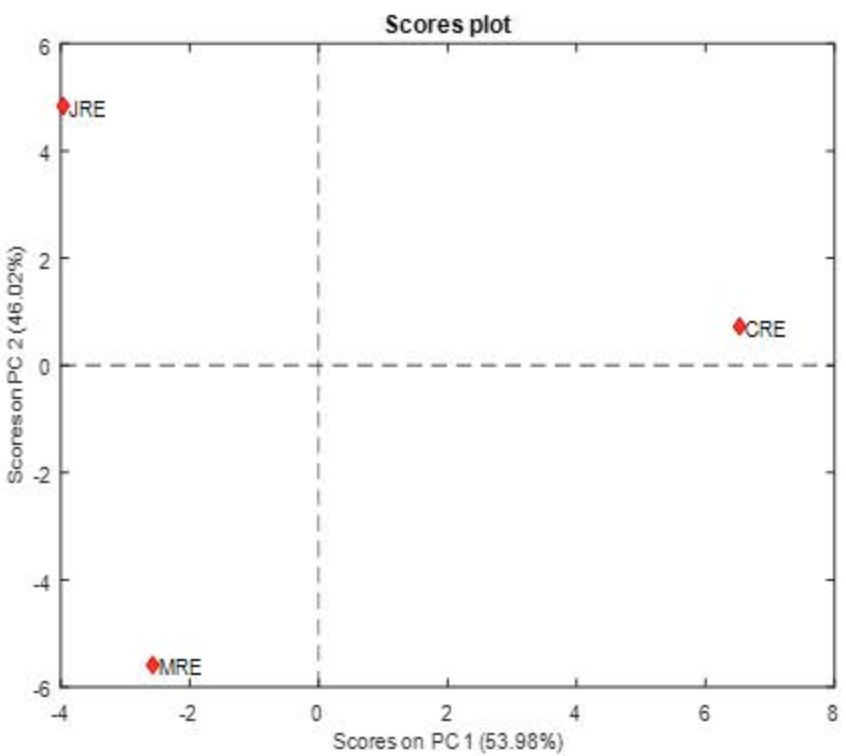
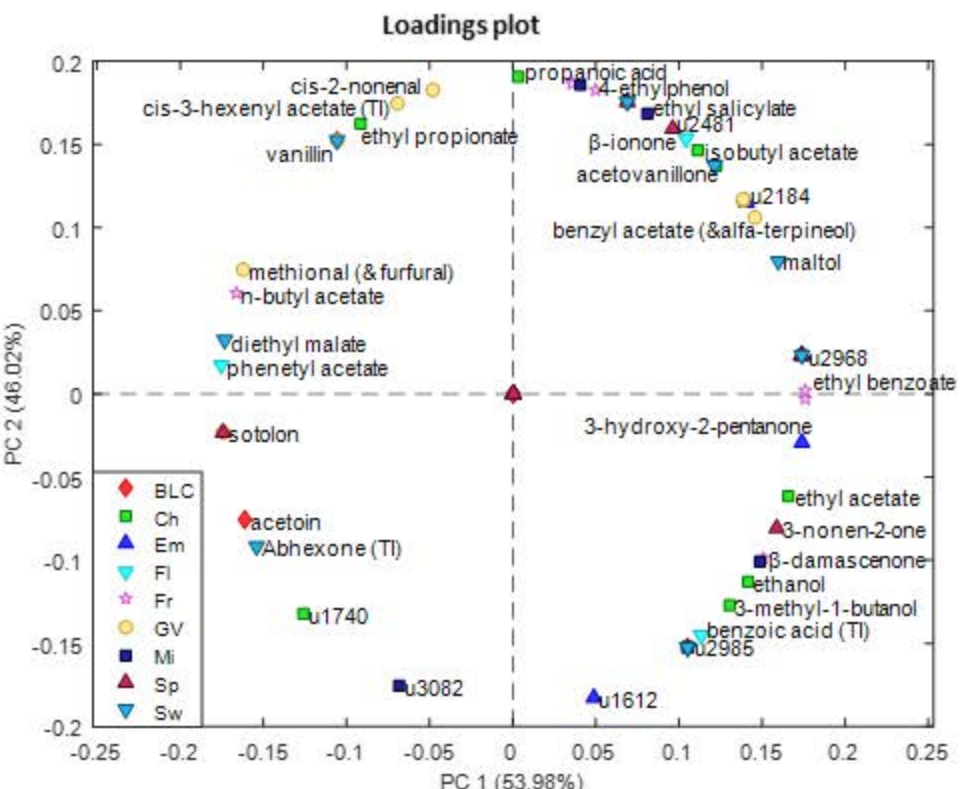
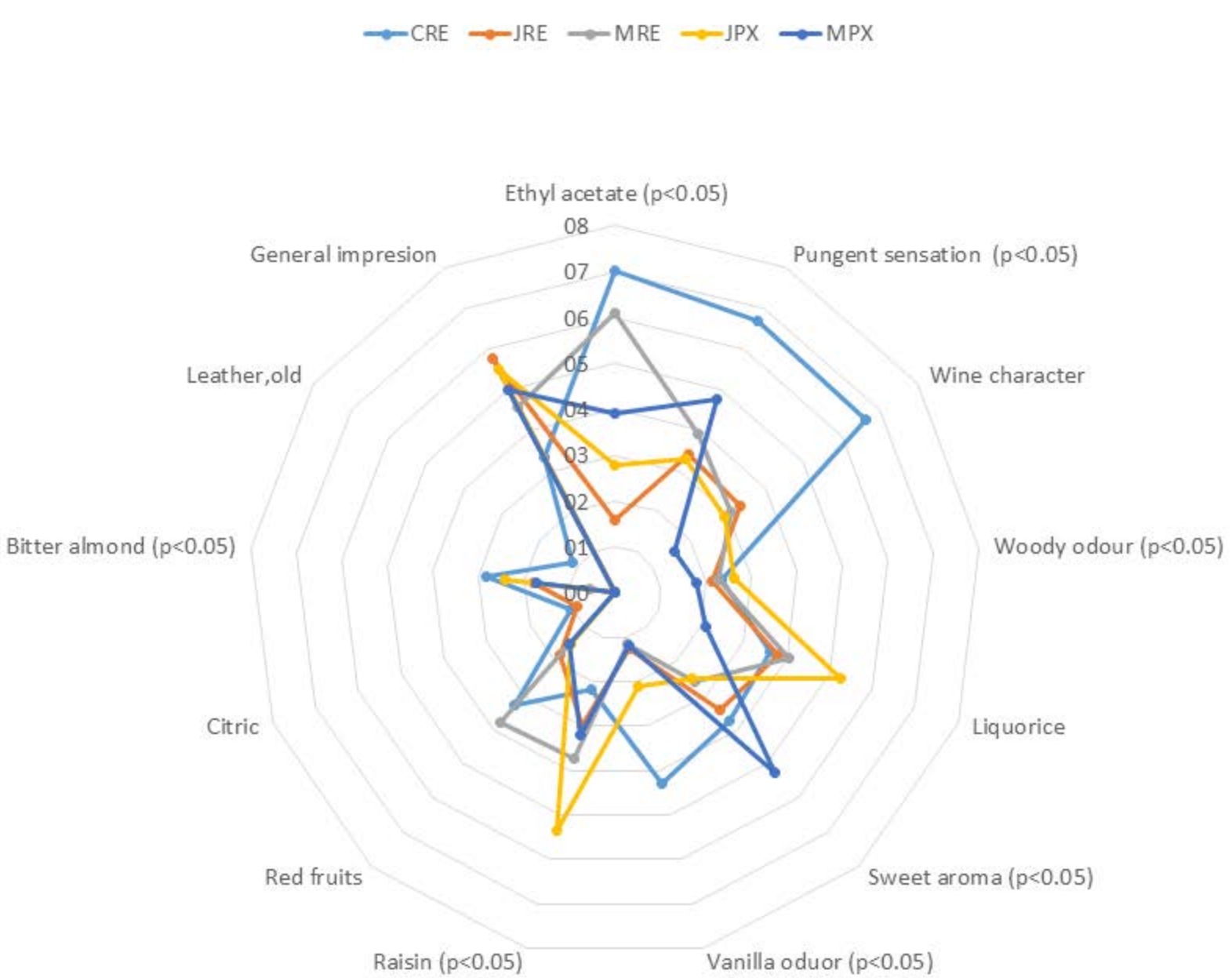
PX
CRE
MPX
JRE
MRE
JPX
Scores on PC 2 (25.68%)
Scores on PC 1 (33.44%)

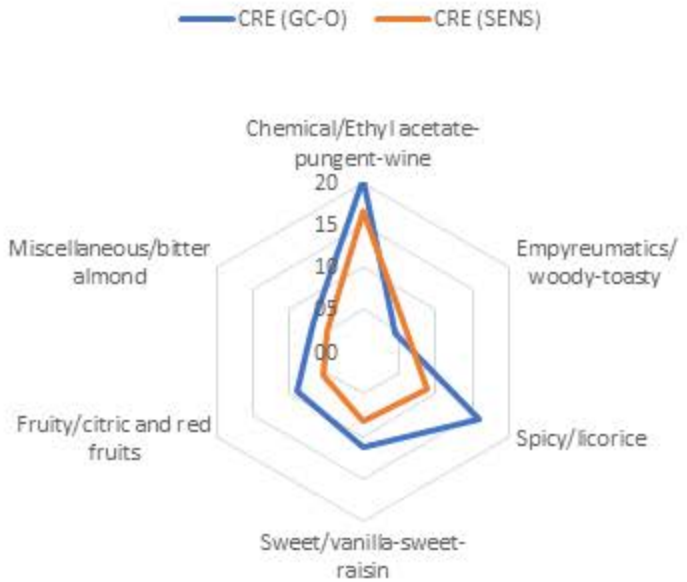

Loadings plot
u2158
u2184
β-ionone
3-methyl-1-butanol
ethyl benzoate
maltol
2,6-dimethylpyrazine
2-methyl-1-butanol
acetaldehyde diethylacetal
ethyl salicylate
3-hydroxy-2-pentanone
pantolactone
ethyl octanoate
phenylacetic acid
β-damascenone
ethanol
ethyl acetate
acetic acid
isobutyl acetate
cis-3-hexenol
cis-β-methyl-γ-octalactone
hexanoic acid
3-nonen-2-one
cis-2-nonenal
p-vinylguaiacol
ethyl isovalerate
octanoic Acid
4-ethylphenol
u2481
vanillin
acetovanillone
4-ethylguaiacol
diacetyl
phenethyl alcohol
u2327
cis-3-hexenyl acetate (TI)
u1612
u2968
Dihydromaltol
phenetyl acetate
methionol
BLC
Ch
Em
Fl
Fr
GV
Mi
Sp
Sw
Abhexone (TI)
diethyl malate
n-butyl acetate
u3082
u1740
acetoin
sotolon
methional (& furfural)
PC 2 (25.68%)
PC 1 (33.44%)

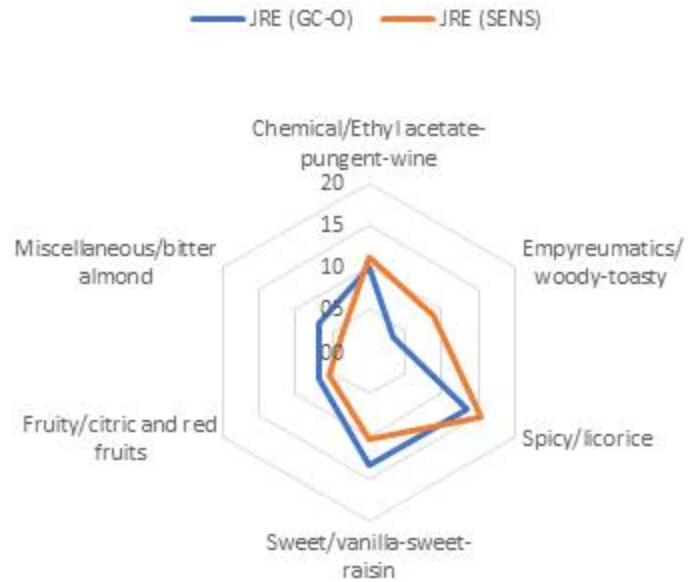
B)

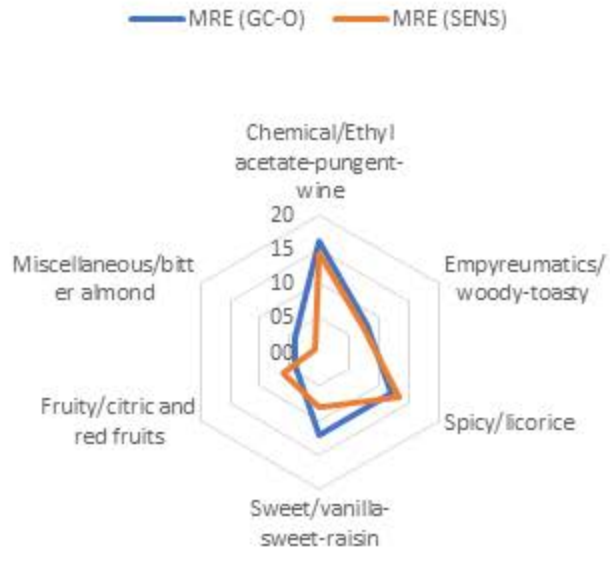

Scores plot
JRE
CRE
MRE
Scores on PC 2 (46.02%)
Scores on PC 1 (53.98%)

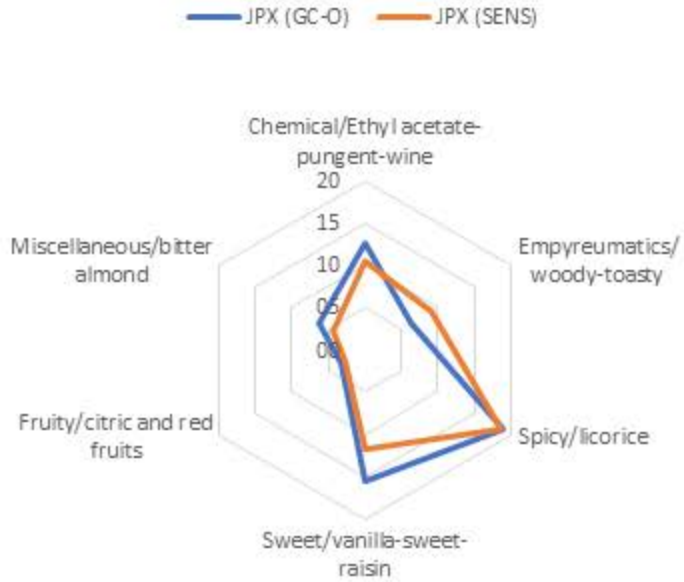

Loadings plot
propanoic acid
cis-2-nonenal
4-ethylphenol
cis-3-hexenyl acetate (TI)
ethyl salicylate
u2481
ethyl propionate
vanillin
β-ionone
isobutyl acetate
acetovanillone
u2184
benzyl acetate (&alfa-terpineol)
maltol
methional (& furfural)
n-butyl acetate
diethyl malate
u2968
phenetyl acetate
ethyl benzoate
3-hydroxy-2-pentanone
sotolon
BLC
Ch
Em
Fl
Fr
GV
Mi
Sp
Sw
ethyl acetate
acetoin
3-nonen-2-one
Abhexone (TI)
β-damascenone
ethanol
u1740
3-methyl-1-butanol
benzoic acid (TI)
u2985
u3082
u1612
PC 2 (46.02%)
PC 1 (53.98%)

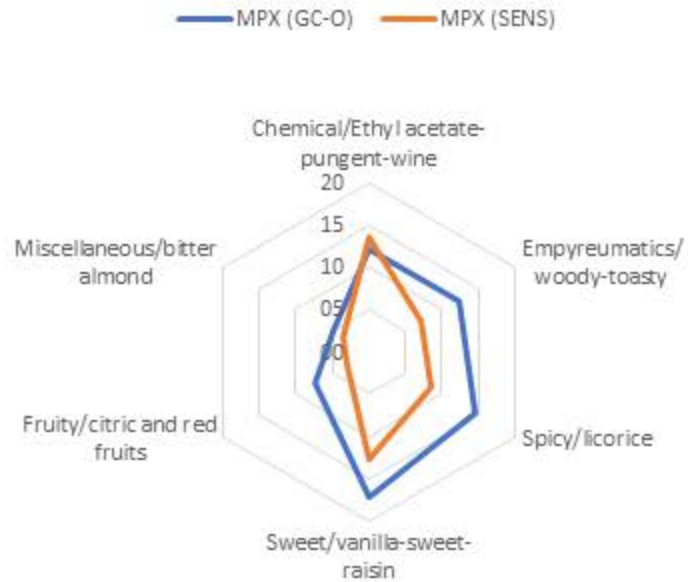

CRE
JRE
MRE
JPX
MPX
Ethyl acetate (p<0.05)
Pungent sensation (p<0.05)
Wine character
Woody odour (p<0.05)
Liquorice
Sweet aroma (p<0.05)
Vanilla oduor (p<0.05)
Raisin (p<0.05)
Red fruits
Citric
Bitter almond (p<0.05)
Leather,old
General impresion
08
07
06
05
04
03
02
01
00


CRE (GC-O)
CRE (SENS)
Chemical/Ethyl acetate-pungent-wine
Empyreumatics/ woody-toasty
Spicy/licorice
Sweet/vanilla-sweet-raisin
Fruity/citric and red fruits
Miscellaneous/bitter almond
20
15
10
05
00


JRE (GC-O)
JRE (SENS)
Chemical/Ethyl acetate-pungent-wine
Empyreumatics/ woody-toasty
Spicy/licorice
Sweet/vanilla-sweet-raisin
Fruity/citric and red fruits
Miscellaneous/bitter almond
20
15
10
05
00


MRE (GC-O)
MRE (SENS)
Chemical/Ethyl acetate-pungent-wine
Empyreumatics/ woody-toasty
Spicy/licorice
Sweet/vanilla-sweet-raisin
Fruity/citric and red fruits
Miscellaneous/bitter almond
20
15
10
05
00


JPX (GC-O)
JPX (SENS)
Chemical/Ethyl acetate-pungent-wine
Empyreumatics/ woody-toasty
Spicy/licorice
Sweet/vanilla-sweet-raisin
Fruity/citric and red fruits
Miscellaneous/bitter almond
20
15
10
05
00


MPX (GC-O)
MPX (SENS)
Chemical/Ethyl acetate-pungent-wine
Empyreumatics/ woody-toasty
Spicy/licorice
Sweet/vanilla-sweet-raisin
Fruity/citric and red fruits
Miscellaneous/bitter almond
20
15
10
05
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BLOQUE IV.

DESARROLLO DE UNA HERRAMIENTA INFORMÁTICA PARA LA AUTENTIFICACIÓN Y CLASIFICACIÓN RÁPIDA DE VINAGRES

CAPÍTULO VIII:
VinegarScan

CHAPTER VIII.

VinegarScan

RESUMEN

Una vez realizado el análisis de los vinagres de vino con DOP, de distintas categorías, así como de vinagres de vino sin DOP, con distintos envejecimientos y orígenes, por distintas metodologías, se seleccionó UV-vis como el método que proporcionaba mejores resultados en términos de clasificación y autentificación teniendo en cuenta todos los tipos de vinagres posibles, con el fin de crear una herramienta informática simple y fácil de usar por cualquier persona. Así, se procede a realizar una herramienta informática o software, implementando los resultados de los modelos jerárquicos de clasificación obtenidos en el capítulo IV (Bloque I), con el fin de que sea capaz de clasificar/autentificar cualquier tipo de vinagre de vino de forma rápida y sencilla, identificando distintos orígenes, métodos de producción, envejecimientos o calidades.

Para ello, además de utilizar los datos espectrales UV-vis y la estructura jerárquica desarrollada en el Capítulo IV, se necesitaron desarrollar nuevos algoritmos de clasificación lateral discriminante (DT) debido al cambio de lenguaje de programación de Matlab a C++.

El software resultante tiene una ventana simple de uso, con tres botones, una zona de representación gráfica y una de resultados. Además, los resultados se muestran de forma visual mediante una representación de una botella típica de vinagre, de distintos colores según la DOP o tipo de vinagre, y llena a distintos niveles según el porcentaje de correcta clasificación.

Debido a esta sencillez y a que permite obtener una clasificación de una muestra de vinagre de una forma muy rápida, esta herramienta podría ser implementada en los laboratorios de control de los Consejos Reguladores, así como para inspectores e incluso podría ser de utilidad para los propios productores, ya que el equipo es portátil y solo necesitaría de un ordenador para realizar un control rutinario de sus propios vinagres.

Este software ha sido inscrito en el Registro de Propiedad Intelectual de la Universidad de Sevilla.

1. COMPONENTES Y METODOLOGÍA

El software diseñado se basa en la información del modelo jerárquico obtenido con los datos espectrales de UV-vis (Capítulo IV). Es decir, contiene los datos espectrales que conforman el set de calibración con sus correspondientes algoritmos de pre-procesado, así como la estructura del modelo jerárquico creada en dicho capítulo. Por tanto, los espectros se pre-procesaron aplicando Variable aleatoria normal tipificada (SVN) y centrado en la media, tal y como se realizó en el capítulo IV del bloque I. Sin embargo, con el fin de poder desarrollar un software libre, sin necesidad del entorno Matlab y que fuese fácilmente exportable e instalable mediante el entorno C++, se desarrollaron nuevos algoritmos de clasificación lateral discriminante (DT). Estos algoritmos DT cuentan con una estructura basada en árbol, y están basados en una herramienta de modelo de decisión que muestra gráficamente el proceso de clasificación a partir de una entrada dada y una vez conocidas las categorías de salida (Drazin and Montag, 2012). Este método es uno de los algoritmos de aprendizaje que genera modelos de clasificación basándose en una estrategia "divide y vencerás" (Safavian y Landgrebe, 1991). El algoritmo se desarrolla creando subconjuntos de datos, descomponiendo el conjunto de datos entero en subconjuntos más pequeños. El modelo final es una estructura de árbol con nodos de decisión y nodos raíz, similar a la mostrada en el capítulo IV de la presente memoria de tesis.

De todos los DT existentes, se ha aplicado el DT J48, que es una modificación del DT C4.5 pero adaptada a la herramienta de técnicas de minería de datos (Witten y Frank, 2005). Este método es bien conocido para el análisis de datos y está considerado uno de los mejores algoritmos para llevar a cabo tareas de clasificación (Anyanwu y Shiva, 2009; Priyam et al., 2013; Wu et al., 2008). El DT J48 se aplicó para desarrollar el modelo con los parámetros que aparecen a continuación: 0.5 como factor de confianza y 5 como tamaño mínimo de registros agrupados.

Para desarrollar los modelos de clasificación se ha utilizado la herramienta de software libre WEKA (descargar en: https://www.cs.waikato.ac.nz/ml/weka/downloading.html, último acceso: Abril 2019). Para desarrollar la aplicación software se ha utilizado la herramienta QtCreator 4.9 bajo el lenguaje de programación C++ y el compilador MigGW 7.3.

2. RESULTADOS

En este caso se desarrolló un modelo jerárquico en el que cada nodo (rule en la imagen) era un DT. El modelo jerárquico es el siguiente figura (Figura 39):

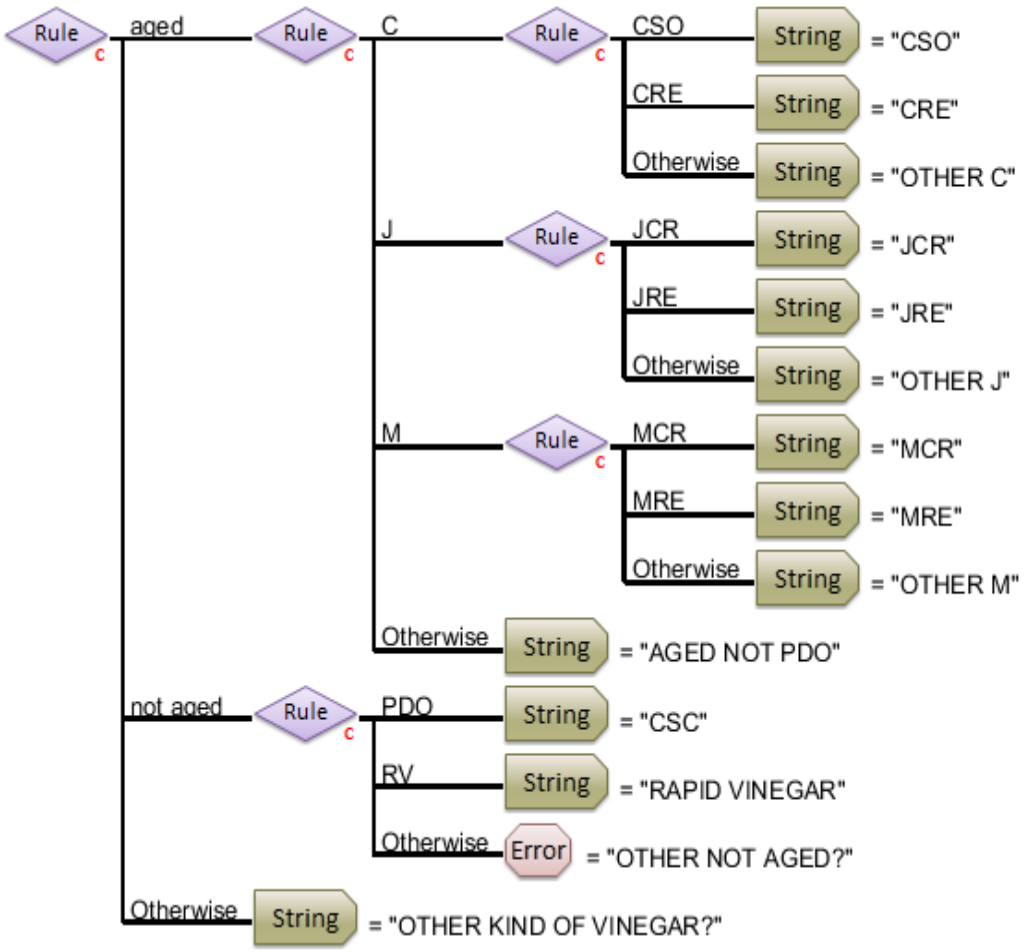

Figura 39. Esquema del modelo jerárquico realizado para el desarrollo de la herramienta o software, igual al del capítulo IV.

El modelo que se ha desarrollado sería el siguiente:

```
wl0044 <= 0.420632
|   wl0915 <= -0.000623: 0
|   wl0915 > -0.000623: 1
wl0044 > 0.420632: 1
------------------------------------------------------------ LEVEL - 1 (AGED/NOT AGED)
wl0088 <= -0.744526: 1
wl0088 > -0.744526
|   wl0069 <= -0.387346: 0
|   wl0069 > -0.387346: 2
------------------------------------------------------------ LEVEL - 2 - AGED (C/J/M)
wl0090 <= 0.48552: 1
wl0090 > 0.48552: 0
------------------------------------------------------------ LEVEL - 2 - NOT AGED (CSC/RV)
wl0192 <= 0.108187: 0
wl0192 > 0.108187: 1
------------------------------------------------------------ LEVEL - 3 - AGED - C (CSO/CRE)
wl0940 <= -0.044134: 1
wl0940 > -0.044134
|   wl0960 <= -0.055108: 1
|   wl0960 > -0.055108
|   |   wl0099 <= -0.798224
|   |   |   wl0001 <= -0.437569: 0
|   |   |   wl0001 > -0.437569: 1
|   |   wl0099 > -0.798224: 0
------------------------------------------------------------ LEVEL - 3 - AGED - J (JCR/JRE)
wl0757 <= -0.071831
|   wl0131 <= 0.263807: 0
|   wl0131 > 0.263807: 1
wl0757 > -0.071831: 1
------------------------------------------------------------ LEVEL - 3 - AGED - M (MCR/MRE)
```

Los resultados de clasificación del modelo son perfectos (100% de clasificación correcta) en todos los casos, si bien, el número de muestras evaluadas para desarrollar el modelo a partir del conjunto de entrenamiento (106), como para el conjunto de validación (38) es un número pequeño, pero suficiente, podría darse el caso de que añadiendo nuevas muestras el modelo fallase y hubiera que evaluar por qué se producen dichos fallos y en qué lugar falla nuestro clasificador.

3. HERRAMIENTA

3.1. Instalación

Una vez creada la herramienta, su instalación es sencilla. Para instalar el programa basta con extraer la información del archivo comprimido "VinegarScan" en la carpeta "C:/" del ordenador tal y como aparece en la Figura 40.

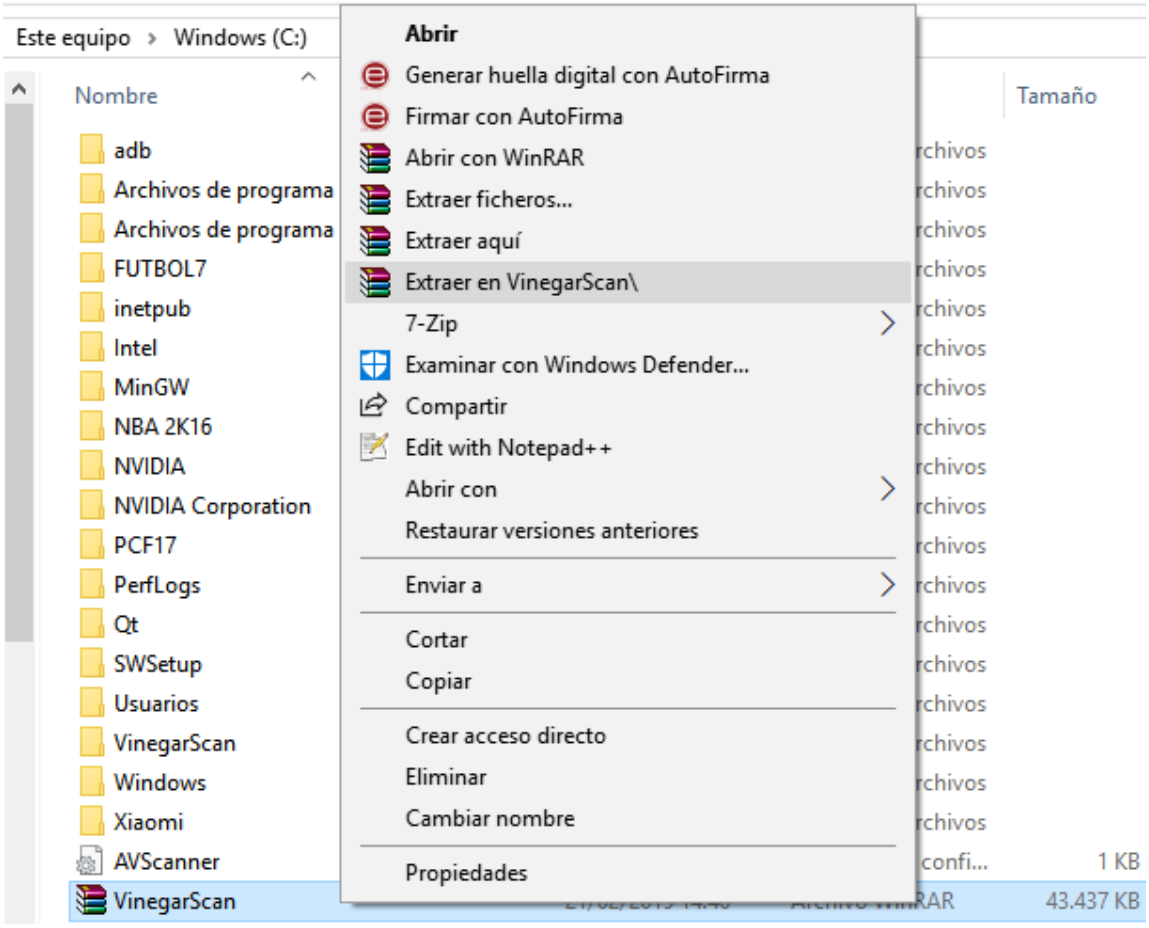

Figura 40. Instalación mediante extracción del archivo comprimido en la carpeta C://

De tal manera que toda la información del software se instalará en nuestro PC en la carpeta "VinegarScan" y contendrá la información que se muestra en la Figura 41.

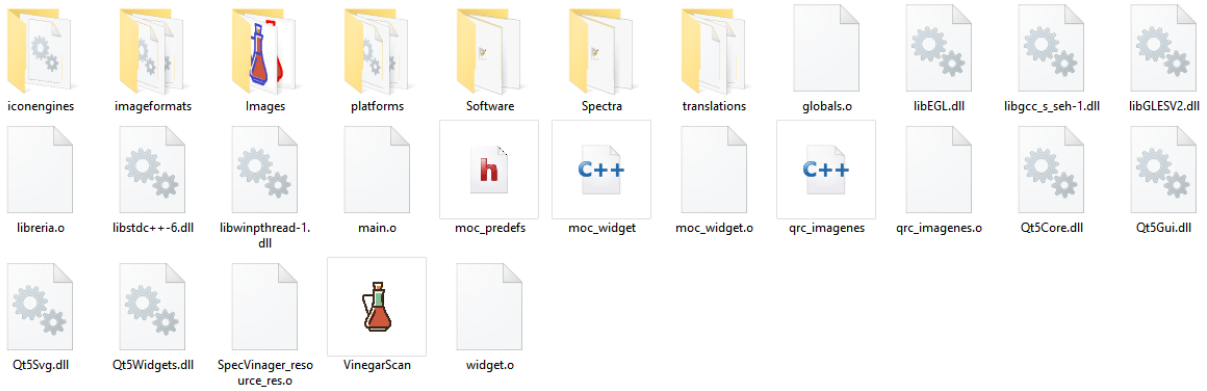

Figura 41. Carpetas y archivos presentes en la ubicación del ordenador "C://VinegarScan" tras la correcta instalación del software.

De todos los directorios instalados destaca el directorio "Spectra" en el cual debemos almacenar los espectros UV-vis que queramos ejecutar en el software. En el podemos encontrar algunos ejemplos de espectros de diferentes vinagres.

3.2. Formato de los espectros

Los espectros UV-vis deben comprender los rangos de longitudes de onda de 280 a 599 nm cada 0.2 nm (es decir, comprender un total de 1566 variables). Esto es debido a que, del total del espectro recogido, como se muestra en el Capítulo IV del Bloque I, se seleccionó este rango ya que proporcionaba la información relevante para las clasificaciones. Para añadir un nuevo espectro, basta con guardar en un Excel los valores de dicho espectro de la siguiente forma:

- una fila únicamente en una hoja de Excel separada.
- sin encabezado de longitudes de las longitudes de onda ni nombres.
- con los decimales delimitados con puntos.

Este fichero Excel se guarda en formato "texto unicode con tabulaciones .txt" y debe ser guardado en el directorio "C:/VinegarScan/Spectra" para mayor comodidad (aunque el software puede abrirlo desde cualquier carpeta en la que se encuentre). La Figura 42 muestra un ejemplo de espectro en formato "*.txt" (ejemplo "s1.txt") y modo de guardarlo.

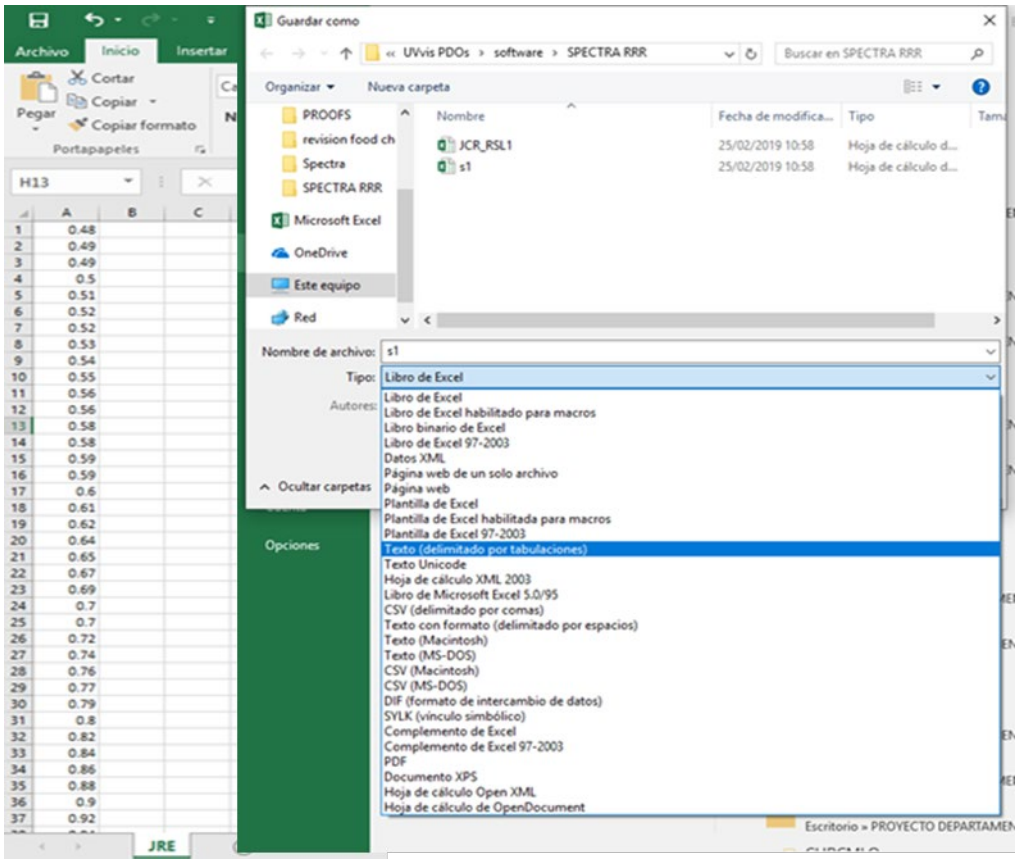

Figura 42. Ejemplo de hoja Excel con espectro UV-vis y el modo de guardarlo en formato “texto .txt”.

3.3. Apariencia y uso del software

Para usar nuestro software basta con hacer doble clic con el botón derecho del ratón sobre el fichero ejecutable del mismo llamado “VinegarScan” y se nos abrirá la aplicación tal y como se muestra en la siguiente figura (Figura 43)**.** Como se puede observar en la figura, la pantalla principal del software comienza mostrando tres botones:

- **Load** para cargar el espectro a analizar. (Sólo uno cada vez).
- **Start** para comenzar el análisis de dicho espectro (inicialmente esta desactivado, ya que no tiene sentido analizar si no hay ningún espectro cargado).
- **Exit** para salir de la aplicación.

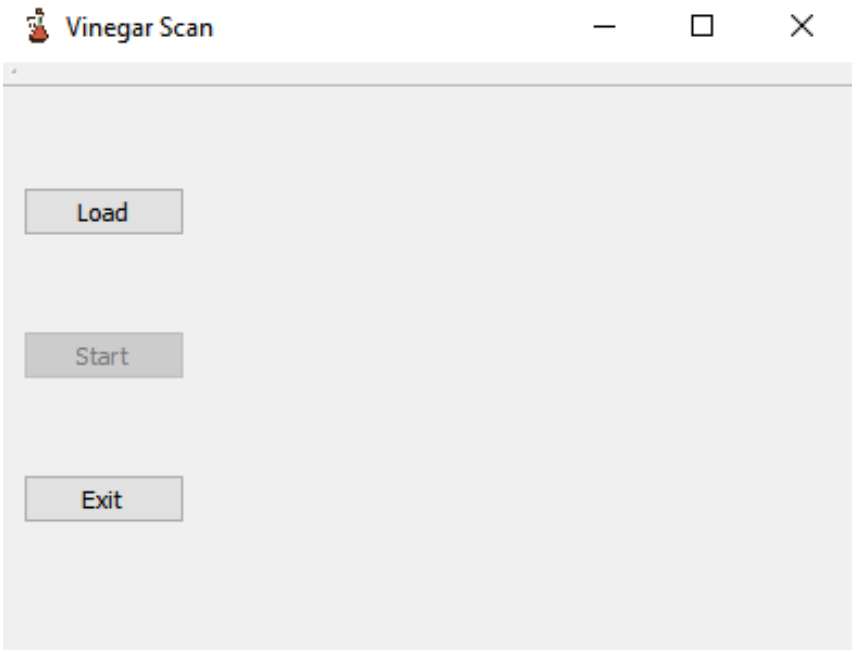

Figura 43. Pantalla inicial del software VinegarScan.

Además, en la barra de estado de Windows se observa el icono de la aplicación tal y como se muestra en la Figura 44 (dicho icono es una imagen libre de derechos de autor y por ello puede ser usada).

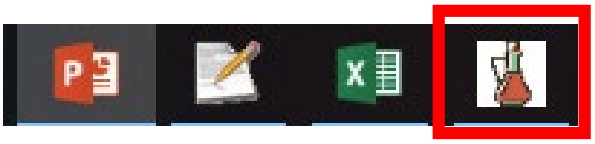

Figura 44. Barra de estado de Windows con el icono de VinegarScan.

Una vez iniciada la aplicación, pulsamos el botón **Load** para cargar un espectro. Tras pulsarlo, aparecerá una pantalla que nos permitirá seleccionar que espectro queremos analizar. Un ejemplo de esto se muestra en la Figura 45, en la que el espectro cargado tiene el nombre de “spec001” y corresponde a un vinagre “Vinagre de Condado de Huelva Reserva”.

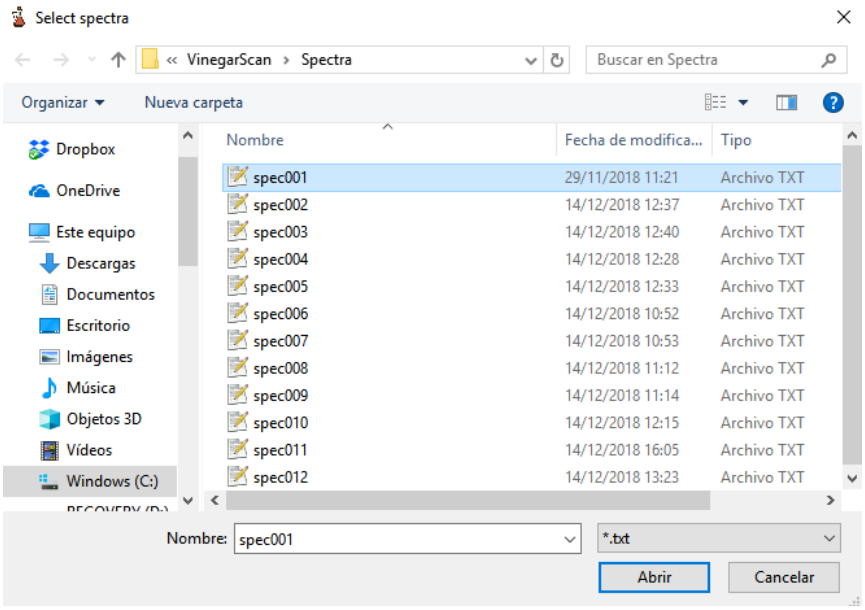

Figura 45. Ejemplo de espectro UV-vis a cargar por el software.

Una vez hemos cargado nuestro espectro se mostrará, en el lado derecho de la pantalla principal, la representación gráfica del mismo y se activará el botón **Start**. Una vez pulsado este botón, aparecerá el resultado en pantalla del análisis de dicho espectro tal y como se muestra en la Figura 46.

Como observamos en la Figura 46, el software ha sido capaz de identificar correctamente el tipo y categoría del vinagre, como “Vinagre de Condado de Huelva Reserva”, mostrando la probabilidad de esta predicción (87.1166%) y una imagen de una botella de vinagre coloreada y más o menos rellena, que nos permita identificar gráficamente que vinagre es y cuál es el porcentaje de probabilidad de que lo sea (en este caso botella de color rojo, casi llena).

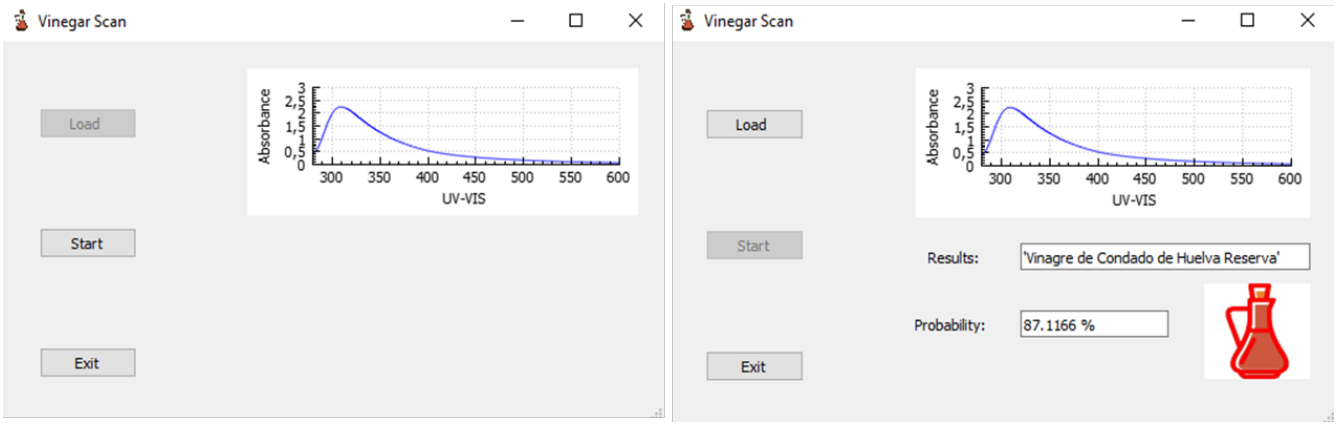

Figura 46. Pantallas del software VinegarScan una vez cargado el espectro (imagen superior) y una vez realizado el análisis al pulsar el botón Start (imagen inferior).

Así, como muestra la siguiente Figura 47, se han establecido cuatro colores distintos para las botellas de vinagre: la botella es roja si el vinagre es de la DOP Vinagre de Condado de Huelva, la botella es verde si el vinagre es de la DOP Vinagre de Jerez, azul si el vinagre es de la DOP Vinagre de Montilla-Moriles y negro para el resto de los casos (vinagres rápidos sin DOP). Así mismo, dependiendo de lo llena que este la botella, podemos identificar gráficamente con que probabilidad de acierto se ha clasificado cierta muestra.

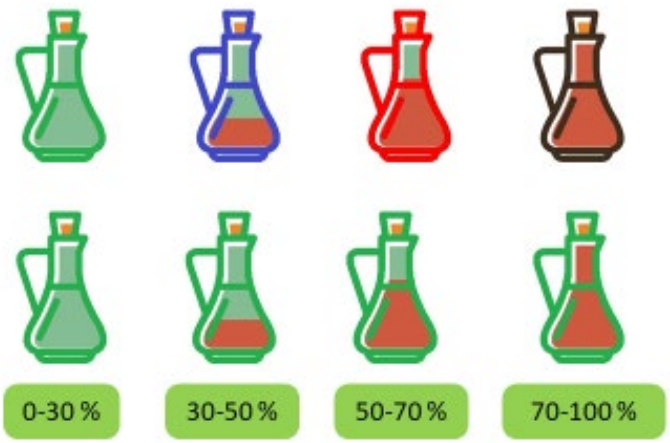

Figura 47. Posibilidades de color y relleno de la representación gráfica de la botella de vinagre resultante del análisis.

Finalmente, una vez se ha realizado el análisis, el botón **Start** se desactiva (ya que no tiene sentido repetir el mismo análisis que acabamos de realizar) y sólo aparece activo el botón **Exit** para salir de la aplicación y **Load** para volver a cargar otro espectro, tal y como muestra la Figura 48.

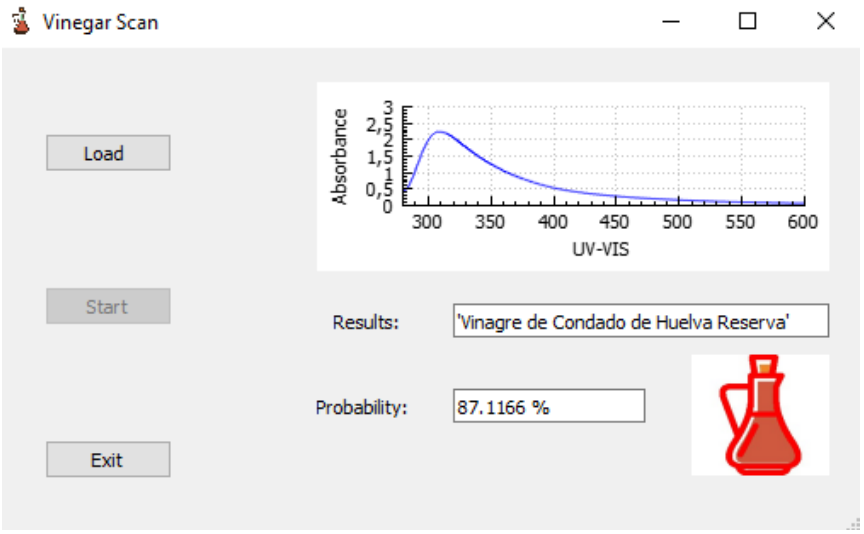

Figura 48. Pantalla final del software, en la que el botón *Start* se inactiva.

3.4. Aplicaciones y expectativas de futuro

Como se ha podido observar en el apartado anterior, esta aplicación tiene unas instrucciones de instalación y uso muy sencillas, necesitando sólo conocimientos básicos de informática. Debido a esta sencillez y a que permite obtener una clasificación de una muestra de vinagre de una forma muy rápida (1 minuto de análisis por UV-vis, 1-2 minutos de exportación de datos y menos de 1 minuto en el uso del software), esta herramienta podría ser implementada en los laboratorios de control de los Consejos Reguladores de las DOPs, así como para inspectores e incluso podría ser de utilidad para los propios productores, ya que el equipo es portátil y solo necesitaría de un ordenador en la bodega para realizar un control rutinario de sus propios vinagres.

Además, esta versión inicial del software se ha protegido mediante su inscripción en el Registro de Propiedad Intelectual de la US. Sin embargo, a pesar de los buenos resultados obtenidos por este software con los datos analíticos que disponíamos, para comprobar su correcto funcionamiento, asegurar clasificaciones más precisas y correctas, se necesita ampliar el número de muestras analizadas, incluyendo más muestras control, así como muestras de otros orígenes, tipos o incluso probándolo con muestras adulteradas. Por otro lado, se están diseñando mejoras para la versión inicial, para mejorar y agilizar su uso y visualización.

4. REFERENCIAS

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5. DISCUSIÓN GENERAL

GENERAL DISCUSSION

FORTALEZAS DE LAS TÉCNICAS ESTUDIADAS EN LA CARACTERIZACIÓN DE VINAGRES Y ANÁLISIS CRÍTICO DE SUS POTENCIALES APLICACIONES Y DEBILIDADES

En el desarrollo de esta tesis se ha tenido en cuenta la complejidad del concepto de caracterización, autentificación y calidad de un producto alimentario con denominación de origen protegida (DOP). Esto es un distintivo de los vinagres de vino objeto de esta tesis, el cual lo diferencia de otros vinagres en los que la calidad apenas tiene presencia en la regulación. Además, el vinagre de vino, y en concreto aquel protegido con una DOP, es una matriz de alta complejidad en cuanto a composición y características organolépticas, así como en cuanto a sus especificaciones en producción, origen, etc. A pesar de que estos vinagres cuentan con unos pliegos de condiciones que regulan numerosos parámetros y condiciones de producción y comercialización, para determinar realmente su calidad, así como para caracterizarlos espectroscópica y aromáticamente de la forma más completa posible, se necesitan evaluar una gran variedad de parámetros, los cuales son también necesarios para conseguir autentificarlos y evitar fraudes.

Hay que tener en cuenta que no todas las técnicas proporcionan la misma información y, por tanto, además de la necesidad de evaluar el nivel de caracterización que permitían, se necesitaba también evaluar la robustez y capacidad de diferenciación de los modelos de clasificación obtenidos por cada técnica espectroscópica, siendo esto uno de los objetivos de la presente tesis. Así, se buscaba conseguir y seleccionar el mejor modelo que permitiese la clasificación de los vinagres de cada DOP, así como de cada categoría, y por tanto su autentificación con respecto a los otros vinagres de vino e incluso a vinagres sin DOP. Y para ello se buscaba utilizar la técnica analítica más rápida, económica y sencilla posible. Un esquema del plan de trabajo del proyecto de tesis seguid, desde su inicio a su finalización, se muestra en la Figura 49. Como se puede observar, la caracterización de los vinagres de vino se dividió en tres bloques principales: caracterización espectroscópica, caracterización isotópica y caracterización aromática. Como se puede observar, la caracterización de los vinagres de vino se dividió en tres bloques principales: caracterización espectroscópica, caracterización isotópica y caracterización aromática. Con respecto a la primera caracterización, se buscaban técnicas rápidas, no destructivas y sin preparación de la muestra que permitieran caracterizar y/o clasificar las diferentes muestras de vinagre de vino según su DOP o su categoría. Se partió de la espectroscopía de infrarrojo medio (ATR-FTIR), y se realizó el análisis del primer conjunto de

muestras de vinagre de vino con DOP del que se disponía. Este estudio permitió la caracterización de las diferentes categorías establecidas en cada DOP mediante el estudio de ciertas bandas espectrales que, en muchas ocasiones, sus intensidades de absorbancia mostraban diferencias significativas entre categorías. Sin embargo, los resultados de clasificación no fueron lo suficientemente satisfactorios, ya que no se conseguía diferenciar muestras según su DOP. Por ello, se empleó en la espectroscopía de infrarrojo cercano (NIR). Todas las muestras anteriores, junto con muestras nuevas que se consiguieron adquirir, así como algunas sin DOP, fueron analizadas por NIR. Este método permitió una mejor diferenciación y clasificación entre categorías de cada DOP, así como entre vinagres con DOP y vinagres sin DOP o vinagres rápidos. Sin embargo, el espectro no proporcionaba apenas información para la caracterización, y la interpretación del mismo requería un mayor tratamiento quimiométrico. Además, la clasificación entre DOPs independientemente de la categoría no fue lo suficientemente adecuada, al igual que ocurría por ATR-FTIR, lo que indicaba que este tipo de clasificación era altamente complejo al tratarse de grupos con composición química y procedimientos de producción no homogéneos (por ejemplo, con diferentes años de envejecimiento).

Tras observar que por estas técnicas no se conseguían buenos resultados de clasificación entre DOPs, se prosiguió el estudio de las muestras mediante espectroscopía de fluorescencia multidimensional (EFM) debido a los resultados satisfactorios obtenidos en un estudio previo de las muestras de vinagre de vino de la DOP Vinagre de Jerez (Callejón et al., 2012). Los resultados obtenidos por EFM permitieron una ligera caracterización de los vinagres de vino de las 3 DOPs, mediante el estudio de los fluoróforos principales que se extrajeron mediante PARAFAC, los cuales no se pudieron identificar de forma individualizada debido a que no solo un compuesto emite a unas longitudes de onda concretas, y a que la naturaleza del alimento y el entorno de los fluoróforos influyen en la señal de fluorescencia. Además, a partir de estos fluoróforos se consiguió una buena clasificación de ciertas categorías dentro de cada DOP mediante máquina de vectores de soporte (alrededor de un 90% de clasificación correcta), así como de las DOP entre muestras de la misma categoría (entre un 82 y un 100% de correcta clasificación). Sin embargo, esta técnica no permitió la clasificación de DOPs independientemente de la categoría a la que pertenecían.

Además, esta técnica, EFM, en combinación con la quimiometría, mostró la capacidad de detectar y cuantificar, por primera vez, la cantidad de caramelo de mosto adicionado a un vinagre de vino, siendo de interés la capacidad de disponer de un método rápido y rentable para

ello, así como de poder establecer un límite o protocolo de control para la adición de este caramelo a vinagres con DOP.

Por otro lado, en la búsqueda del mejor modelo de clasificación se probó también el análisis de las muestras de vinagre de vino con DOP por ^{1}H-RMN. Esta técnica permite la identificación de ciertos compuestos que marcan la diferencia entre muestras. Por tanto, resulta muy útil para la caracterización química de las mismas. Los modelos de clasificación de categorías y de DOPs obtenidos mediante esta técnica fueron medianamente buenos (entre el 75 y el 100% de las muestras fueron correctamente clasificadas según la DOP). Sin embargo, esta técnica no es sencilla, rápida, ni económica y se necesitan profesionales expertos en la técnica para realizar los análisis e interpretar los resultados, así como para el tratamiento quimiométrico de los datos (alineación, integración, etc.).

Siguiendo con el objetivo de buscar el mejor modelo de clasificación que fuese capaz de diferenciar entre distintas DOPs y categorías, se probó la combinación y fusión de los datos obtenidos por las técnicas espectroscópicas anteriormente citadas. La fusión de estos datos mostró mejores resultados de clasificación entre DOPs, independientemente de la categoría, (entre un 91.7 y un 100% de correcta clasificación), que los modelos de clasificación basados en los análisis espectroscópicos individuales. Además, mediante la fusión de datos se pudo estudiar de manera simple la complementariedad y diferencias en la información espectral recogida por cada una de las técnicas. Sin embargo, a pesar de la buena clasificación obtenida y las ventajas que supone la fusión de datos, la principal desventaja es que el análisis de las muestras por distintas técnicas espectroscópicas carece de aplicación práctica de forma rutinaria al requerir el desarrollo de varios métodos, tratándose por tanto de una estrategia de análisis de alto coste.

Por este motivo se optó por probar la espectroscopía de UV-vis, la cual, a pesar de no proporcionar mucha información específica sobre diversos componentes de la muestra, sí que contaba con las ventajas de ser una de las técnicas más sencillas, rápidas y económicas de entre las diferentes técnicas espectroscópicas. Además, en este caso los análisis se realizaron mediante un equipo portátil, con el fin de obtener un procedimiento que permitiera la implementación en un futuro en bodegas u organismos de control. A pesar de que esta técnica no permitía una profunda caracterización, fue la técnica que proporcionó los mejores modelos de clasificación (100% de correcta clasificación), siendo capaz de diferenciar: (1) el método de producción de un vinagre de vino, tradicional o rápido, o lo que es lo mismo, vinagres con DOP de vinagres sin DOP; (2) el tiempo de envejecimiento o categorías; (3) vinagres de vino de diferentes DOP; (4) así como las diferentes categorías dentro de cada DOP. Aparte de los

resultados de clasificación satisfactorios, las principales ventajas de este procedimiento son la rapidez y simplicidad en el análisis, sin requerir técnicas quimiométricas complejas de pretratamiento de los datos. Sin embargo, además de la difícil identificación de compuestos, presentó el inconveniente de que fue la única técnica que requirió una manipulación previa de la muestra, aunque simple, ya que fue necesaria la dilución del vinagre de vino con agua, con el fin de evitar saturaciones de la señal. Debido a los excelentes resultados obtenidos por esta técnica, junto con su sencillez, precio, rapidez, capacidad de portabilidad y de crear modelos jerárquicos de clasificación robustos, se decidió construir la herramienta informática de clasificación de vinagres con los datos de UV-vis, siendo éste el último objetivo de esta tesis.

Por otro lado, junto con todas las técnicas espectroscópicas citadas, las muestras también fueron analizadas mediante técnicas isotópicas, cromatografía de gases acoplada a espectrometría de masas y olfatometría (GC-MS y GC-MS-O), así como mediante análisis sensorial. Las técnicas isotópicas permitieron controlar ciertos parámetros relacionados con el fraude y origen, diferenciando muestras con DOP de muestras sin DOP de distintas localizaciones geográficas. Con respecto al análisis cromatográfico, en primer lugar, se determinó el perfil volátil de los vinagres de vino con DOP y sus categorías, mediante una evaluación y selección previa del método de extracción de compuestos volátiles más adecuado. Así, la técnica de extracción seleccionada fue la extracción por sorción en espacio en cabeza estático (HSSE), debido a que extraía el mayor número de compuestos relevantes, así como por el perfil volátil que proporcionaba y sus ventajas. Una vez seleccionada la técnica de extracción, las muestras de vinagre de vino con DOP, así como las distintas categorías de envejecimiento y dulzor, fueron analizadas por GC-MS, permitiendo diferenciar las DOPs según un perfil volátil característico de cada una, así como seleccionar marcadores químicos responsables de esta diferenciación. Por otro lado, se realizó la caracterización aromática de las muestras mediante GC-MS-O y análisis sensorial, consiguiéndose diferenciar las diferentes DOPs y sus correspondientes categorías mediante una serie de marcadores aromáticos, denominados odorantes de impacto, los cuales son los principales responsables de las diferencias y características sensoriales de estos vinagres.

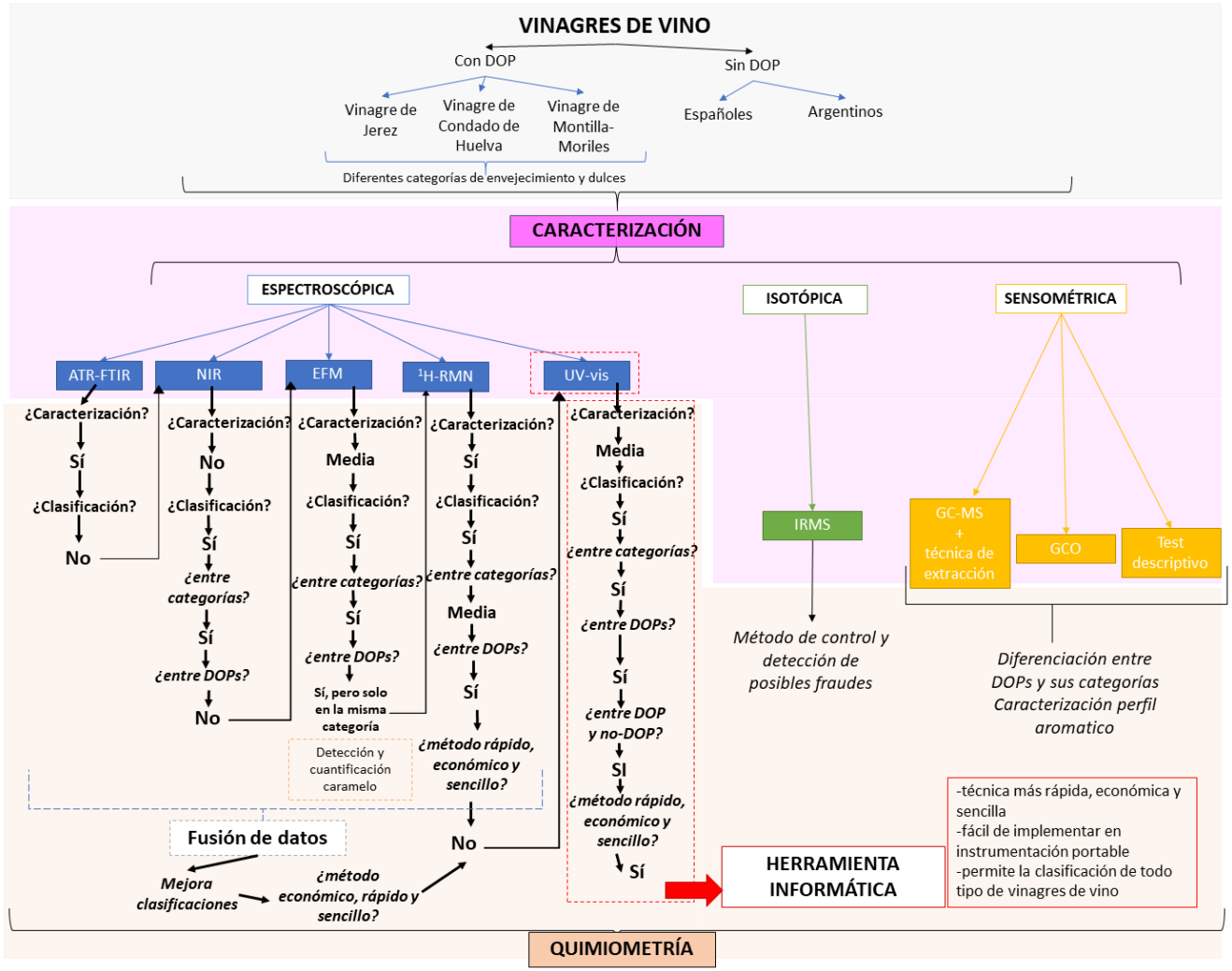

Figura 49. Esquema resumen del plan de trabajo para el logro de los objetivos de la presente Tesis Doctoral.

La selección de nuevas herramientas de análisis desarrolladas para su aplicación en vinagres de vino influye positivamente en la caracterización de la calidad de este producto. Cada una de estas técnicas y la información analítica que proporciona ofrece la posibilidad de controlar uno o varios aspectos de la calidad global del vinagre de vino. Es decir, nos proporciona una mejor y más completa caracterización, y, por tanto, facilita su autentificación y diferenciación. Pero dependiendo del objetivo o propósito que busquemos, según los resultados obtenidos, se ha visto que unas técnicas son más adecuadas que otras. Las técnicas empleadas, junto con sus características, ventajas e inconvenientes se describen en la **Tabla 8.**

Así, si lo que buscásemos fuera una diferenciación rápida de una muestra de vinagre de vino de otra, sin intención de conocer composición de la muestra, que pudiera realizarla cualquier persona sin necesidad de muchos conocimientos previos, de forma rápida, rutinaria y económica, la técnica de UV-vis sería la más adecuada. Si, por otro lado, quisiésemos conocer algo más de la diferencia entre muestras de vinagre de vino de distinta DOP o categoría, como un perfil general o huella dactilar de cada tipo de vinagre, sin profundizar demasiado, y realizando análisis rápidos y económicos, sería más adecuada la espectroscopía de fluorescencia multidimensional o la espectroscopía de infrarrojo medio, dependiendo del nivel de conocimientos quimiométricos del analista. Ambas técnicas ofrecen la posibilidad de diferenciar las categorías de las DOP según el tiempo de envejecimiento o dulzor mediante observación a simple vista de los resultados (espectro o matriz de excitación-emisión), sin embargo, por EFM se requeriría el manejo de más técnicas quimiométricas para el pretratamiento y extracción de información (como por ejemplo PARAFAC), mientras que por MIR se puede ver a simple vista, sin pretratamiento de los espectros, un aumento o disminución de ciertas bandas según el envejecimiento, e incluso un perfil característico de los vinagres de vino Pedro Ximénez, aunque la clasificación no se consiguiese.

Si por otro lado nos centráramos en uno de los principales indicadores de la calidad de un alimento, como es el aroma, y quisiéramos determinar marcadores químicos que diferencien las muestras de vinagres de vino con DOP, así como que se relacionasen con las diferencias o características sensoriales de los vinagres, partiríamos de la cromatografía de gases-espectrometría de masas (GC-MS), implementándola con análisis olfatométricos, los cuales también han dado buenos resultados en términos de autentificación y diferenciación, aunque sean técnicas menos rápidas, menos económicas, con necesidad de reactivos y tratamiento tedioso de los datos, así como de analistas expertos.

Tabla 8. Técnicas y características, ventajas e inconvenientes en su uso para la caracterización de vinagres de vino españoles con DOP.

Técnica	Información analítica	Herramientas quimiométricas empleadas	Ventajas	Potencial en tareas de clasificación y control	Inconvenientes y aspectos críticos
ATR-FTIR	Bandas fundamentales y huella dactilar en el rango de infrarrojo medio	PCA PLS-DA	- Metodología analítica rápida, no destructiva y de bajo coste. - Sin manipulación/preparación de la muestra. - No contaminante. - Interpretación sencilla de los espectros. - No requiere técnicas quimiométricas complejas. - Proporciona más detalles sobre los tipos de moléculas presentes que NIR. - Perfiles espectrales muy sensibles a la composición química (incluso las moléculas muy similares pueden producir espectros muy distintivos).	- Diferenciación de las categorías establecidas en cada DOP de vinagre de vino, incluso a simple vista de los espectros. - Definición de bandas puntuales relevantes para cada categoría en la región de la huella dactilar.	- No se consigue una correcta clasificación de las categorías, DOP o tipos de vinagre de vino (alrededor de 58.4% de correcta clasificación). - Difícil identificación de compuestos específicos. - Requiere limpieza exhaustiva del cristal ATR.
NIR	Huella dactilar en el rango de infrarrojo cercano	SMT + SNV PCA PLS-DA	- Metodología analítica rápida, no destructiva y de bajo coste. - Sin manipulación/preparación de la muestra. - No contaminante. - Disponibilidad de equipos portables. - Suministra información simultánea de diferentes parámetros de calidad.	- Diferenciación y clasificación de las categorías establecidas dentro de cada DOP (desde 86.7-100% de correcta clasificación). - Diferenciación y clasificación de vinagres de vino sin y con DOP (entre 95.8 y 100% de correcta clasificación).	- Requiere experiencia para la interpretación de los espectros. - Requiere técnicas quimiométricas para obtener resultados. - Las bandas de absorción son muy amplias y se superponen (muestras químicamente diferentes pueden dan lugar a perfiles espectrales casi indistinguibles). - Difícil identificación de compuestos específicos

					- Presenta bandas menos resueltas que MIR. - No se consigue una correcta clasificación de DOPs.
EFM	Fluoróforos y huella dactilar de fluorescencia	PARAFAC PLS-DA o SVM PCA PLS NPLS NPLS-DA	- Alta sensibilidad. - Técnica sencilla y económica. - No contaminante. - Sin manipulación/preparación de la muestra. - Permite tanto la toma de huellas dactilares, como la medición cuantitativa de sustancias que contienen fluoróforos. - Útil para muestras complejas, como el vinagre de vino, con más de una especie emisora. - Permite cuantificaciones con precisión.	- Clasificación de las categorías establecidas dentro de cada DOP de vinagre de vino (entre 37.5 y 100% de correcta clasificación con PLS-DA y entre 91.7 y 100% con SVM). - Clasificación correcta del 85.7-100% entre DOPs considerando categorías similares. - Detección y cuantificación de caramelo de mosto en las muestras de vinagres de vino.	- Efecto de la dispersión Raleigh y bandas de Raman que puede dar lugar a la aparición de picos fantasma - Necesidad de técnicas quimiométricas para la extracción, identificación y cuantificación de compuestos en matrices complejas. - Saturación con muestras de colores oscuros. - No clasifica correctamente DOP y no DOPs. - No clasifica correctamente las muestras Reserva dentro de una DOP o entre DOPs.
^{1}H-RMN	Espectro de protones	PCA LDA-PLS-DA	- Metodología analítica rápida y no destructiva. - Poca manipulación de la muestra (adición de patrón interno). - No contaminante. - Suministra información simultánea de diferentes parámetros de calidad.	- Clasificación correcta de entre el 75 y 100% entre DOPs independientemente de la categoría. - Diferenciación de las categorías establecidas en cada DOP de vinagre de vino. - Identificación de algunos compuestos relevantes para la diferenciación.	- Técnica analítica de alto coste. - Requiere experiencia del analista. - Requiere experiencia para la interpretación y manipulación de los datos. - No clasifica perfectamente las DOPs.

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UV-vis	Huella dactilar en el rango UV-vis	PCA SIMCA o PLS-DA (HCM y Bootstrapping)	- Metodología analítica rápida y no destructiva. - Poca manipulación de la muestra (dilución con agua MilliQ). - No contaminante. - Todas las moléculas orgánicas son capaces de absorber la radiación electromagnética en esa zona del espectro. - Permite cuantificaciones con precisión. - Disponibilidad de equipos portables. - Permite la clasificación jerárquica y su implementación en una herramienta informática.	- Clasificación correcta del 100% de las categorías establecidas en cada DOP de vinagre de vino. - Clasificación correcta del 100% entre DOPs. - Clasificación correcta del 100% de vinagres de vino con DOP y sin DOP. - Clasificación correcta del 100% según envejecimiento. - Clasificación correcta del 100% según método de producción (sumergido o superficial).	- Saturación con muestras de colores oscuros. - Difícil identificar específicamente compuestos.
análisis de isótopos estables $\delta^{13}C$ y $\delta^{18}O$ por IRMS	Huella dactilar isotópica C-O	ANOVA-test LSD Coeficiente de correlación de Pearson	- Permite encontrar la trazabilidad del producto y a su vez detectar la trazabilidad del compuesto con el cual se ha realizado el fraude. - Permite determinar el origen, la pureza y procedencia del producto de una forma rápida y eficaz. - No requiere técnicas quimiométricas complejas para la interpretación de los datos.	- Distinción de vinagres de vino españoles de distinta latitud (norte y sur) e incluso de distintas regiones de la misma latitud (las 3 DOP). - Control de adulteraciones: fuente de ácido acético (materiales de partida) y presencia de agua externa a la de las uvas.	- Instrumentación cara. - Se necesitan valores de referencia. - Los valores se afectan por parámetros climatológicos, almacenamiento, etc.
HSSE-GC-MS	Perfil volátil	MCR ANOVA PCA Heatmap	- La combinación de GC-MS con técnicas quimiométricas como MCR mejora los resultados obtenidos, facilita y acelera el procesado de datos y disminuye problemas comunes asociados a estos análisis.	- Clasificación y caracterización de los vinagres de vino con DOP y sus categorías. - Determinación de ciertos compuestos volátiles como marcadores de cada DOP.	- El perfil volátil obtenido depende en gran medida de la técnica de extracción o muestreo utilizada. - Técnica analítica cara y compleja.

			- HSSE permite la extracción de un mayor número de compuestos volátiles que HS-SPME o DHS. - Permite obtener mucha información sobre la composición química de la muestra.		- Requiere el uso de reactivos y analistas con conocimientos previos. - Requiere preparación de la muestra.
GC-MS-O + MF + OAVs	Perfil aromático	PCA	- Técnica analítica más apropiada para la determinar el impacto real de los compuestos volátiles presentes en el vinagre que contribuyen al aroma de un vinagre (aromas activos). - Potente herramienta para la caracterización de aromas en vinagre.	- Determinación de los compuestos volátiles que contribuyen realmente al aroma general percibido de la muestra (odorantes de impacto). - Diferenciación de los vinagres de vino con DOP y sus categorías según sus odorantes de impacto y notas aromáticas características.	- Limitaciones respecto a los efectos sinérgicos y antagónicos de los odorantes y la ley psicofísica de percepción. - Distintas técnicas olfatométricas con distintas ventajas e inconvenientes (dilución, tiempo-intensidad, de frecuencia de impacto y de frecuencia modificada). - Requiere manipulación de la muestra.
QDA	Perfil sensorial	ANOVA	- Permite evaluar la calidad sensorial del vinagre desde el punto de vista del productor o consumidor. - No requiere manipulación de la muestra.	- Diferenciación de las categorías envejecidas o dulces de los vinagres de vino con DOP mediante atributos sensoriales diferenciales.	- Análisis arduo debido a sabor y olor agresivo del ácido acético. - Necesidad de seleccionar atributos sensoriales concretos. - Necesita un panel sensorial entrenado. - Necesidad de disponer de numerosos estándares para el entrenamiento.

Gracias al análisis de las muestras de vinagre de vino por todas estas técnicas, se ha podido lograr una caracterización multiparamétrica muy completa a distintos niveles (perfil o huella digital, composicional, sensorial y aromático) de cada una de las DOPs de vinagres de vino, así como de sus categorías establecidas. Todos los parámetros evaluados y obtenidos por las distintas técnicas para cada una de las DOPs y sus categorías se han resumido en las siguientes tablas (**Tabla 9, Tabla 10 y Tabla 11**). Con ello podría decirse que se ha logrado caracterizar, a distintos niveles, tanto las categorías establecidas en cada DOP de vinagre de vino, como una caracterización general de cada DOP, lo cual permite a su vez, diferenciarlas, clasificarlas y autentificarlas, así como detectar fraudes.

En general, cabe destacar que en las muestras de la DOP Vinagre de Jerez presentaron bandas de absorción en el rango de UV-vis de mayor intensidad que las otras DOPs con máximos a 310 nm en las muestras menos envejecidas y a 330 y 360 nm en las muestras más envejecidas, mientras que sus valores medios de $\delta^{18}O$ (1.91 ± 1.11‰) fueron intermedios entre la DOP Vinagre de Condado de Huelva y Vinagre de Montilla-Moriles. Como compuestos volátiles destacables para esta DOP se podría decir que sus muestras, independientemente de la categoría, mostraron una mayor presencia de acetato de *trans*-2-hexenilo, *trans*-2-hexen-1-ol y 5-hidroximetilfurfural (5-HMF) junto con cis-2-nonenal, a diferencia de las otras dos DOPs. Con respecto a su perfil aromático, presentó un mayor número de odorantes de impacto con notas aromáticas verdes y vegetales, además de algo florales y especiadas en esta DOP, y principalmente en sus vinagres de vino de la categoría Reserva. Además, su categoría Pedro Ximénez destacó por la presencia de compuestos volátiles de la familia de los acetatos y ácidos, así como por una banda característica en la región espectral de 1175-1000 cm^{-1}, junto con la región espectral 3.22-5.26 ppm de RMN y fluoróforos que emiten a 550–570/600–650 nm ex/em. Todos estos parámetros se relacionaron con la mayor presencia de azúcares, caramelo de mosto y compuestos de Maillard. Esta categoría dulce presentó un mayor número de odorantes de impacto responsables de notas aromáticas más especiadas y licorosas.

Con respecto a la DOP Vinagre de Montilla-Moriles, presentaron la menor intensidad en las bandas de absorción en el rango de UV-vis respecto a las otras dos DOPs, con máximos a 295 nm las menos envejecidas y a 300 nm las más envejecidas, además de los valores medios más bajos de $\delta^{18}O$ (1.91 ± 1.11‰) con respecto a las otras dos DOPs. En relación a los compuestos volátiles característicos de esta DOP destacarían el diacetilo y la acetoína, junto con 2,3-heptanona y 3-etoxi propanoato de etilo, entre otros. La composición aromática de los vinagres de vino de esta DOP, principalmente de la categoría Reserva, presentó un mayor número de odorantes de impacto con notas aromáticas de la familia de los lácteos (mantequilla, queso),

junto con algunas químicas. Los vinagres de vino pertenecientes a su categoría dulce, Pedro Ximénez, presentaron unas bandas características en la región del espectro alrededor de 1175-1000 cm^{-1}, como ocurre con los vinagres de la misma categoría de la DOP Vinagre de Jerez, pero en este caso fueron más intensas y marcadas, siendo esta diferencia reconocible a simple vista. Además, también presentaron bandas de excitación-emisión a 530-605/585-655 nm ex/em relacionadas con fluoróforos como azúcares, caramelo de mosto o furfurales, pero en este caso menos intensas que los Pedro Ximénez de la DOP Vinagre de Jerez. Como marcadores destacaban diacetilo, metional y furfural, así como una gran proporción de acetatos. Estos vinagres dulces además presentaron una mayor presencia de odorantes de impacto con notas aromáticas empireumáticas (aromas tostados, ahumados) y dulces que las diferencian de los de la otra DOP.

Finalmente, los vinagres de vino de la DOP Vinagre de Condado de Huelva presentaron, en general, una mayor presencia de ácido acético y etanol, según muestran las bandas del espectro alrededor de 1410, 1290 y 1045 cm^{-1} y las regiones de RMN de 1.18, 3.6, y 2.1 ppm. Además, esta DOP presentó una intensidad de bandas de absorción en el rango del UV-vis intermedia entre las otras dos DOPs, con máximos a 350 nm para las menos envejecidas y a 330 nm para las más envejecidas, mientras que, por otro lado, presentó en general, los mayores valores medios de $\delta^{18}O$ (2.67± 1.45‰) respecto a las otras dos DOPs. Además, es la única DOP que comercializa vinagres de vino sin envejecimiento (CSC), los cuales destacaron por su mayor presencia en agua (bandas características a 1500 y 5200 cm^{-1}) y bandas de absorción con máximo a 290 nm con la menor intensidad de entre todas las muestras analizadas. Dentro de la composición volátil y aromática, destacan, en esta DOP, compuestos como el nonanoato de metilo, eucaliptol y 1-heptanol, así como el acetaldehído dietilacetal entre otros. Estos vinagres presentaron un mayor número de odorantes de impacto con notas aromáticas químicas y afrutadas que los vinagres de las otras dos DOPs, así como presentó una mayor sensación punzante, vinosa y a pegamento de sus muestras.

Como se puede ver en estos resultados, hay ciertas características aromáticas, perfiles o huellas espectrales y compuestos químicos específicos, que podrían servir como marcadores diferenciales de cada una de las DOPs, así como de sus categorías.

Tabla 9. Caracterización de las muestras de vinagre de vino de la DOP Vinagre de Jerez mediante las distintas técnicas analíticas empleadas en la presente tesis doctoral. * Muestra sin analizar.

VINAGRE DE JEREZ PDO				
	JCR	**JRE**	**JPX**	**VJ**
ATR-FTIR (1500-900 cm-1)	~1500 cm^{-1} (agua) y ~1015 cm^{-1} (etanol, glicerol)	~1085 cm^{-1} y ~1045 cm^{-1} (alcoholes, aldehídos, y algunos ácidos, esteres y éteres). Picos mayor altura que JCR	1175-1000 cm^{-1} menos intensos y marcados que MPX (azúcares y compuestos de Maillard)	1150-1000 cm^{-1} + ~1045 cm^{-1} + ~1250 cm^{-1}
NIR (12000-4000 cm^{-1})	~5200 cm^{-1} (agua)	5200-6500 cm^{-1} (compuestos aromáticos y fenólicos)	~5600 cm^{-1} (azúcares, fructosa y glucosa)	~5500 cm^{-1} + ~6200 cm^{-1} + 11000-12000 cm^{-1}
EFM	Máximo a 370/450 nm ex/em (fenoles, flavonoles y vinatminas). Mayores valores para los fluorórofos F1-F4	Máximo a 370-470/470–550 nm ex/em (5-HMF, caramelo de mosto). Disminuyen los valores de los fluoróforos con respecto a JCR.	Máximo a 550–570/600–650 nm ex/em (similar a las bandas asociadas al caramelo de mosto, azúcares de uva, furfurales y compuestos de Maillard). Altos valores para F5 en JCR y JPX.	F1: 465/535 nm ex/em (vit B2, FMN, FAD) F2: 400/480 nm ex/em F3: 500/580 nm ex/em (pigmentos marrones) F4: 350/440 nm ex/em F5: 585/655 nm ex/em (relacionada con los PX)
UV-VIS	Máximo a 310 nm y de 400-500 nm	Máximo a 330 nm con hombro a 360 nm. Importancia del rango visible 500-600 nm (color por envejecimiento)	*	La mayor intensidad (2.5 a.u.) de las tres DOPs y máximo a 330-360 nm
ISOTOPOS	1.83±1.04‰ $\delta^{18}O$ -25.04±0.46‰ $\delta^{13}C$	3.18 ± 1.06‰ $\delta^{18}O$ -25.13±0.5 ‰ $\delta^{13}C$	*	Valores medios de $\delta^{18}O$ intermedios entre las otras DOPs: 1.91 ± 1.11‰
^{1}H-RMN	0.95 ppm, 1.5 ppm, 2.1 ppm (ácido acético), 2.2-2.3 ppm (butanone, acetone, acetoacetate), 2.8-3.0 ppm (ácido malico y citrico)	0.9 ppm (2-hidroxi-3-methilvalerato), 1.1 ppm (propionato), 1.2 ppm (etanol), 1.3 ppm (acetoina), 1.4 ppm (2-fenilpropionato), 1.5 ppm (alanine), 3.3 ppm, 8.3 (ácido fórmico) y 9.6 ppm.	3.22-5.26 ppm (glucosa, fructosa, azúcares en general, menos relación que en MPX)	Malato, glutarato, n-acetilglutamato (2.4ppm) y ácido fórmico (8.26ppm). Azúcares (3.22-5.26 ppm).
GC-MS	Acetato de trans-2-hexenilo, Trans-2-hexen-1-ol	Trans-2-hexen-1-ol, 5-HMF	3-heptanol, β-damascenona, isovalerato de geranilo, guaiacol, 5-HMF, Acetato de trans-2-hexenilo, trans-2-hexen-1-ol, hexadecanoato de metilo, 2-heptanol,	β-damascenona, 5-HMF, 3-heptanol, acetate de trans-2-hexenilo, Trans-2-hexen-1-ol

			2-cyclopentanona, ácido 2-metilpropanoico y ácido 3-metilbutanoico. ACETATOS: acetate de metilo, acetate de etilo, acetate de 2-etilhexilo, 3-oxobutan-2-yl acetato y acetato de bencilo.	
Odorantes de impacto por GC-MS-O según MF y OAV	*	Propionato de etilo, octanoato de etilo, ácido propanoico, acetato de feniletilo, 4-etilfenol	Diacetilo, metional y furfural	metional y furfural ácido isobutírico
Odorantes de impacto por GC-MS-O según MF	*	cis-2-nonanal, acetate de cis-3-hexenilo	6,7-dihydro-7-hydroxylinalool	
Aroma GC-MS-O	*	Vegetal-verde, floral, especiado	Especiado, dulce y floral	Verde y floral
QDA	*	Destaca la alta impresión general	Destaca el caracter licoroso y alta impresión general	Destaca la alta impresión general

Tabla 10. Caracterización de las muestras de vinagre de vino de la DOP Vinagre de Montilla-Moriles mediante las distintas técnicas analíticas empleadas en la presente tesis doctoral. * Muestra sin analizar.

VINAGRE DE MONTILLA-MORILES PDO				
	MCR	**MRE**	**MPX**	**VMM**
ATR-FTIR (1500-900 cm^{-1})	~1500 cm^{-1} (agua) y ~1015 cm^{-1} (etanol, glicerol)	~1045 cm^{-1} (alcoholes, aldehídos, y algunos ácidos, esteres y éteres). Pico mayor que MCR y menor que en JRE	~1175-1000 cm^{-1} más intensa y marcados que JPX (Azúcares y compuestos de Maillard)	995-1150 cm^{-1} principalmente por los PX.
NIR (12000-4000 cm^{-1})	5200 cm^{-1} (agua)		~5600 cm^{-1} mayor intensidad en MPX que JPX (azúcares)	~5200 y ~6500 cm^{-1} (azúcares de uva, furfurales, y compuestos de Maillard)+ 11000-12000 cm^{-1}
EFM	Elevados valores para todos los fluoróforos, excepto para el F5. Destaca el F1.	Disminuyen los valores de los fluoróforos, excepto para F3 y F5.	Elevados valores para F5.	F1: 375/460 nm ex/em, (cumarinas, taninos, fenoles, flavonoles) F2: 410/500 nm ex/em F3: 470/550 nm, ex/em (vitB2 y derivados) F4: 340/420 nm ex/em F5: 530/605 nm ex/em (relación con PX)
UV-VIS	Máximo a 290 nm y entre 350-450 nm	Máximo a 300 nm. Importancia del rango visible 500-600 nm (color por envejecimiento)	*	La menor intensidad de todas las DOPs y máximo a 295-310 nm
ISOTOPOS	1.56±2.35‰ $\delta^{18}O$ -24.78±0.96‰ $\delta^{13}C$	2.82 ± 1.27‰ $\delta^{18}O$ -25.03±063‰ $\delta^{13}C$	*	Los menores valores medios de $\delta^{18}O$ (1.60 ± 2.62‰) de todas las DOPs.
^{1}H-RMN	1 ppm (butanona), 1.06 ppm(isobutirato), 2.1 ppm (ácido acético), 2.16 ppm (acetoina), 1.2 y 3.6 ppm (etanol), 3.3 y 4.6 ppm (beta-glucosa), 5.3 ppm (sacarosa)	9.49 ppm (acetoximetilfurfural), 6.8 ppm, 4.7 ppm, 4.52 ppm (ácido tartárico), 2.6 ppm, 2.25 ppm (acetona), 1.79 ppm (6-acetilglucosa), 1.15 ppm (isopropanol).	De 3 a 5 ppm (región de los azúcares).	5-HMF (4.7+9.45ppm) y azúcares (3.2-5.25ppm)
GC-MS	Diacetilo, acetoína, 3,4-dihydroxy-3,4-dimethyl-	2- y 3-heptanone	3-etoxi propanoato de etilo, canfeno, 2-metil-1-hexadecanol, 1-octen-3-ol,	diacetilo, acetoína, 3-etoxipropanoato de etilo, 2- y 3-heptanona, 2-metil-1-

	2,5-hexanediona, acetato de acetoina. Ácidos 2-metilbutanoico, octanoico y decanoico		benzotiazol, acetoína y diacetilo.	hexadecanol, 1-octen-3-ol, p-cresol y canfeno
Odorantes de impacto por GC-MS-O según MF y OAV	*	Acetoína	Actato de feniletilo y vanillina	Acetoina
Odorantes de impacto por GC-MS-O según MF	*	Abhexona	Diacetato de 2,3-butanediol, 2,6-dimetilpiracina, dihidromaltol, ciclohexanoato de etilo, 3-nonen-2-ona, β-damascenona, p-vinilguaiacol y ácido benzoico	ácido fenilacético
Aroma GC-MS-O	*	Mantequilla-láctico-queso, químico	Dulce, empirumático y especiado	Empirumático
QDA	*	Destaca el atributo acetato de etilo y frutos rojos	Destaca el atributo dulce	Dulce, frutos rojos y acetato de etilo

Tabla 11. Caracterización de las muestras de vinagre de vino de la DOP Vinagre del Condado de Huelva mediante las distintas técnicas analíticas empleadas en la presente tesis doctoral. * Muestra sin analizar.

VINAGRE DE CONDADO DE HUELVA PDO				
	CSC	**CSO**	**CRE**	**VC**
ATR-FTIR (1500-900 cm^{-1})	~1500 cm^{-1} (agua) y ~1015 cm^{-1} (etanol, glicerol)	~1045 cm^{-1} y ~1085 cm^{-1} (alcoholes, aldehídos, y algunos ácidos, esteres y éteres), picos altura media	~1045 cm^{-1} y ~1085 cm^{-1} (alcoholes, aldehídos, y algunos ácidos, esteres y éteres), picos más altos	~1410, ~1290 y 1045 cm^{-1} (Ácido acético y etanol)
NIR (12000-4000 cm^{-1})	~5200 cm-1 (agua)		~5800 cm^{-1} y ~6200 cm^{-1}	~6000 cm^{-1} + ~8000 cm^{-1}
EFM	Pico máximo a las menores longitudes de onda (370/440 nm ex/em). Destacables altos valores para los fluoróforos F1 y F3.	Aumentan los valores de F2 y F4 con respecto a CSC, disminuyendo los valores para F1 y F3.	Con el envejecimiento, aumentan los valores para F4, y tendiendo a 0 los valores de F3.	F1: 370/470 nm ex/em (cumarinas, taninos, fenoles, flavonoles) F2: 420/505 nm ex/em F3: 300/425 nm ex/em (compuestos fenólicos) F4: 485/560 nm ex/em (compuestos fenólicos) F5: no hay
UV-VIS	La menor intensidad de todas (aprox. 1 a.u.) y máximo a 290 nm. Forma del espectro distinta.	Máximo a 350-450 nm	Máximo a 330 nm. Importancia del rango visible 500-600 nm (color por envejecimiento)	Intensidad intermedia entre las 3 DOPs y máximo a 330 nm
ISOTOPOS ^{18}O y ^{13}C		2.70 ±1.31‰ $\delta^{18}O$ -25.09±1.01‰ $\delta^{13}C$	3.69 ± 2.08‰ δ18O -25.02±0.44‰ δ13C	Los mayores valores de $\delta^{18}O$ (2.67 ± 1.45‰) de las tres DOPs
^{1}H-RMN	1.04 ppm (isobutirato), 2.0 ppm (acetamida), 2.1 ppm (ácido acético), 2.16 ppm (acetoina).	1.03 ppm (isobutirato), 2.0 ppm (acetamida), 2.3 ppm (acetoacetato), 2.8-3.01 ppm (ácidos cítricos y málicos)	6.7-9.4 ppm (5-HMF, ácido fórmico), 2.6 ppm (ácido succínico y β-alanina), 1.06-1.3 ppm (etanol, isopropanol y acetato de etilo).	Isopropanol (1.13ppm), etanol (1.18+3.6ppm), acetato de etilo (1.25 ppm), ácido acético (2.1ppm), β-alanina y succinatos (2.6ppm)
GC-MS	Metilnonanoato, trans-2-decenal, eucaliptol, safranal	Ácido 2-metilbutanoico, 1-heptanol, α-terpineol, eucaliptol, octanal, 2-hidroxi-2-ciclopenten-1-ona. Más esteres de etilo y acetales en general.	Nonanoato de metilo	1-heptanol, nonanoato de metilo, ácido 2-metilbutanoico, 2,2,6-trimetil-ciclohexanona, trans-2-decenal, eucaliptol y α-terpineol. Benzaldeido,

				ácido propanoico y decanoico, butirato de etilo.
Odorantes de impacto por GC-MS-O según MF y OAV	*	*	acetaldehído dietil acetal, acetato de isobutilo, isovalerato de etilo, guaiacol	
Odorantes de impacto por GC-MS-O según MF	*	*	Etanol, acetato de etilo, 3-metil-1-butanol, salicilato de etilo, β-ionona y maltol	
Aroma GC-MS-O	*	*	Químico, afrutado y especiado	Químico y afrutado
QDA	*	*	Sensación punzante, olor a acetato de etilo, carácter vinoso y afrutado	Sensación punzante, olor a acetato de etilo, carácter vinoso y afrutado

6. CONCLUSIONES

CONCLUSIONS

CONCLUSIONES

De los resultados obtenidos durante el desarrollo de la presente Tesis Doctoral se extraen las siguientes conclusiones:

1. Respecto a la caracterización y clasificación espectroscópica e isotópica se puede concluir que:

1.1. Se ha realizado la caracterización espectroscópica de los vinagres de vino españoles con DOP y de sus categorías comercializadas mediante ATR-FTIR, NIR, EFM, ^{1}H-RMN, y UV-vis, obteniéndose, por primera vez, una huella dactilar de cada tipo de vinagre, eficaz para su diferenciación y clasificación.

1.2. El análisis de las muestras por ATR-FTIR permitió la caracterización y diferenciación de las categorías comercializadas en cada DOP mediante las regiones del espectro alrededor de: ~1500 cm^{-1} (asociada a la presencia de agua) y ~1015 cm^{-1} (asociada a la presencia de etanol o glicerol) para las categorías menos envejecidas; bandas más intensas alrededor de ~1085 cm^{-1} y ~1045 cm^{-1} (asociadas a la presencia de alcoholes, aldehídos, y algunos ácidos, esteres y éteres) para las categorías con mayor tiempo de envejecimiento; y bandas intensas y características en la región del espectro 1175-1000 cm^{-1} (asociada a la presencia de azúcares y compuestos de Maillard), relacionadas con la categoría Pedro Ximénez.

1.3. El análisis de las muestras por NIR permitió una mejor diferenciación y clasificación que ATR-FTIR entre categorías de cada DOP, así como entre vinagres con DOP y sin DOP, mediante la región del espectro alrededor de ~5500 cm^{-1} relacionada con la categoría Pedro Ximénez y asociada a la presencia de azúcares, fructosa y glucosa; una banda a ~6200 cm^{-1} relacionada con las categorías más envejecidas y la presencia de compuestos aromáticos y fenólicos; y una banda alrededor de ~5200 cm^{-1} relacionada con las categoría menos envejecidas y con la mayor presencia de agua en la muestra.

1.4. Los resultados obtenidos por EFM junto con PARAFAC y SVM, permitieron una cierta caracterización de los fluoróforos de los vinagres de vino de las 3 DOPs y una buena clasificación de algunas de sus categorías dentro de cada DOP (alrededor de un 90% de clasificación correcta), así como de las DOPs considerando muestras de la misma categoría (entre un 82 y un 100% de correcta clasificación). La categoría Pedro Ximénez fue la mejor clasificada (100%) debido a la presencia de fluoróforos con bandas de excitación/emisión característicos a 550-570/600-650 nm en la DOP Vinagre de Jerez, y a 530-605/585-655 nm ex/em en la DOP Vinagre de Montilla-Moriles, relacionados con la presencia de azúcares, caramelo de mosto o furfurales.

1.5. Además, EFM en combinación con técnicas quimiométricas, demostró la capacidad de detectar y cuantificar con un alto coeficiente de correlación (R2> 0.921) y alta capacidad de predicción, por primera vez y de forma rápida y económica, la cantidad de caramelo de mosto adicionada a un vinagre de vino.

1.6. Los vinagres de vino españoles con DOP, y sus categorías, fueron analizadas por primera vez por 1H-RMN, obteniéndose una huella dactilar útil para clasificar los vinagres de vinos según DOP, con porcentajes de clasificación correcta mayores al 75%. Las regiones del espectro alrededor de 3.22-5.26 ppm, relacionadas con la presencia de azúcares, fueron relevantes para la categoría Pedro Ximénez, mientras que para las categorías de menor envejecimiento destacó la región alrededor de 0.95 a 2.5 ppm relacionada con la presencia de ácido acético y acetoína.

1.7. La fusión de datos de nivel medio y la fusión de datos basada en el análisis predictivo de pesos comunes y específicos de bloques múltiples (P-ComDim) han sido comparadas por primera vez utilizando los datos espectroscópicos obtenidos por las técnicas anteriormente citadas, y empleándolas por primera vez en dos tipos de matrices, de 2D y 3D. Además, se ha aplicado por primera vez P-ComDim, método recientemente propuesto, en una matriz de datos 3D. Gracias a ello se consiguió una caracterización espectroscópica más completa de los vinagres de vino españoles con DOP además de una correcta clasificación del 90-100% de las muestras según la DOP, independientemente de la categoría a la que perteneciesen.

1.8. Los espectros UV-vis de los vinagres de vino analizados mostraron diferencias observables a simple vista y responsables de su correcta clasificación: Los vinagres de vino de la DOP Vinagre de Jerez de las categorías envejecidas se caracterizaron por bandas UV-vis de mayor intensidad que las otras DOPs con máximos a 310 nm (las menos envejecidas) y a 330 y 360 nm (las más envejecidas), mientras que las categorías envejecidas de la DOP Vinagre de Montilla-Moriles presentaron la menor intensidad en las bandas de absorción en el rango de UV-vis respecto a las otras dos DOPs, con máximos a 295 nm (las menos envejecidas) y a 300 nm (las más envejecidas). Además, los vinagres de vino de la DOP Vinagre de Condado de Huelva presentaron bandas de UV-vis a intensidades intermedias entre las otras dos DOPs, con máximos a 350 nm (las menos envejecidas) y a 330 nm (las más envejecidas), además de a 290 nm y la menor intensidad observada para la categoría sin envejecer.

1.9. El análisis de las muestras por espectroscopía de UV-vis proporcionó los mejores modelos de clasificación (100% de correcta clasificación de todas las muestras), pudiéndose construir un modelo jerárquico de clasificación capaz de diferenciar: el método de producción de un vinagre de vino, tradicional o rápido o lo que es lo mismo vinagres con DOP de vinagres

sin DOP; el tiempo de envejecimiento o categorías; vinagres de vino de diferentes DOP; así como las diferentes categorías dentro de cada DOP.

1.10. El análisis de isótopos estables del carbono ($\delta^{13}C$) permitió indicar si el ácido acético y los azúcares presentes en los vinagres de vino estudiados procedían verdaderamente de la uva, mientras que el análisis isotópico de oxígeno ($\delta^{18}O$) permitió discriminar a todos los vinagres españoles según las tres coordenadas geográficas (latitud, longitud y altitud), asociándose los valores positivos a vinagres del sur (es decir, los vinagres de vino con DOP) y los valores negativos con vinagres del norte (sin DOP). Además, se observaron valores de $\delta^{18}O$ diferentes entre las 3 DOPs, siendo los valores más bajos observados los de los vinagres de vino de la DOP Vinagre de Montilla-Moriles (1.91 ± 1.11‰), intermedios para los de la DOP Vinagre de Jerez (1.91 ± 1.11‰) y más elevados para los de la DOP Vinagre de Condado de Huelva (2.67± 1.45‰).

1.11. Los resultados de clasificación obtenidos y las metodologías estudiadas han permitido desarrollar una herramienta informática clasificatoria de vinagres de vino, llamada "VinegarScan", implementando el modelo jerárquico de clasificación obtenido con los datos de espectroscopía UV-vis. Este método cuenta con la ventaja de haber sido realizado mediante un equipo portátil que permite análisis rápidos, sencillos y económicos. Por ello, se pretende la implementación de esta herramienta, actualmente ya inscrita en el Registro de Propiedad Intelectual de la US, en bodegas u organismos de control de los vinagres de vino.

2. Respecto a los resultados obtenidos por la caracterización aromática de los vinagres de vino españoles con DOP y sus categorías comercializadas mediante HSSE-GC-MS, GC-MS-O y análisis sensorial, se extraen las siguientes conclusiones:

2.1. Tras la evaluación de tres técnicas de extracción para el análisis de los vinagres de vino con DOP mediante GC-MS, se seleccionó la técnica de extracción por sorpción en espacio en cabeza estático (HSSE) debido a que extraía un mayor número de compuestos, permitía una mejor diferenciación de las DOPs y categorías y contaba con más ventajas que otras técnicas estudiadas (tiempo, precio, tiempo de uso, etc.).

2.2. El perfil volátil obtenido por HSSE-GC-MS de los vinagres de vino de cada DOP y de cada una de sus categorías fue diferente y característico para cada una de ellas, permitiendo clasificar correctamente las DOPs, así como seleccionar ciertos compuestos volátiles como marcadores responsables de esta diferenciación, entre los que se podrían destacar *trans*-2-hexenilo, *trans*-2-hexen-1-ol y 5-hidroximetilfurfural (5-HMF) para los vinagres de la DOP Vinagre de Jerez; diacetilo, acetoína, y 2- y 3-heptanona para la DOP Vinagre de Montilla-Moriles, y 1-heptanol, nonanoato de metilo, eucaliptol y α-terpineol para la DOP Vinagre de Condado de Huelva.

2.3. La caracterización aromática de las muestras mediante GC-MS-O, permitió diferenciar las tres DOPs mediante una serie de marcadores aromáticos, denominados odorantes de impacto, seleccionados según los resultados de frecuencia modificada (FM) y los valores de actividad aromática (OAV). Así, los odorantes de impacto para los vinagres Reserva de la DOP Vinagre de Jerez fueron propionato de etilo, octanoato de etilo, ácido propanoico, acetato de fenetilo y 4-etilfenol, y diacetilo, metional y furfural para la categoría Pedro Ximénez. Con respecto a la categoría Reserva de la DOP Vinagre de Montilla-Moriles, los odorantes de impacto seleccionados fueron acetoína y abhexona, mientras que para los Pedro Ximénez fueron el acetato de feniletilo y vainillina. Los odorantes de impacto para la categoría Reserva de la DOP Vinagre de Condado de Huelva según FM y OAV fueron acetaldehído dietil acetal, acetato de isobutilo, isovalerato de etilo y guaiacol.

2.4. La caracterización aromática por GC-MS-O y el análisis sensorial mostraron las diferencias sensoriales y notas aromáticas características de cada uno de estos vinagres. Los vinagres de la DOP Vinagre de Jerez mostraron un mayor porcentaje de odorantes de impacto responsables de aromas verdes y herbáceos, y de notas dulces, especiadas y licorosas para su categoría Pedro Ximénez; los vinagres de la DOP Vinagre de Montilla-Moriles mostraron un mayor porcentaje de odorantes de impacto responsables de aromas a mantequilla-láctico-queso, y de aromas dulces, tostadas y especiadas para la categoría Pedro Ximénez; y los vinagres de la DOP Vinagre de Condado de Huelva mostraron un mayor porcentaje de odorantes de impacto responsables de aromas químicos, punzantes y afrutados.

3. **El conocimiento generado a través de la caracterización realizada de cada una de las DOPs españolas de vinagre de vino ha puesto de manifiesto la calidad diferencial de los vinagres de vino españoles con DOP con respecto a otros vinagres, así como la variedad en calidades dentro de cada DOP. Las diferencias observadas son lo suficientemente notables para desarrollar métodos de control que permitan la autentificación y verificación de las categorías establecidas dentro cada DOP.**

CONCLUSIONS

From the results obtained during the development of the present Doctoral Thesis, the following conclusions are drawn:

1. Regarding the spectroscopic and isotopic characterization and classification, it can be concluded that:

1.1. The spectroscopic characterization of the Spanish PDO wine vinegars and their commercialized categories by means of ATR-FTIR, NIR, EFM, ^{1}H-NMR, and UV-vis has been carried out, obtaining, for the first time, a fingerprint of each type of wine vinegar effective for their differentiation and classification.

1.2. The analysis of the samples by ATR-FTIR enabled characterizing and differentiating the categories commercialized in each PDO through the regions of the spectrum around:~1500cm^{-1} (associated with the presence of water) and ~1015 cm^{-1} (associated in the presence of ethanol or glycerol) for the less aged categories; more intense bands around ~1085 cm^{-1} and ~1045 cm^{-1} (associated with the presence of alcohols, aldehydes, and some acids, esters and ethers) for the categories with the longest aging time; and intense and characteristic bands in the spectrum region 1175-1000 cm^{-1} (associated with the presence of sugars and Maillard compounds), related to the Pedro Ximénez category.

1.3. The analysis of the samples by NIR provided a better differentiation and classification than ATR-FTIR, between categories of each PDO, as well as between vinegars with PDO and without PDO, through the spectrum region around ~5500 cm^{-1} related to the Pedro Ximénez category and associated to the presence of sugars, fructose and glucose; a band at ~6200 cm^{-1} related to the most aged categories and the presence of aromatic and phenolic compounds; and a band around ~5200 cm^{-1} related to the less aged category and with the greater presence of water in the sample.

1.4. The results obtained by EFM in combination with PARAFAC and SVM, provided a certain characterization of the wine vinegar's fluorophores of the 3 PDOs and good classification results for some of their categories within each DOP (around 90% of correct classification), as well as for the PDOs considering samples of the same category (between 82 and 100% of correct classification). Pedro Ximénez category was the best classified (100%) due to the presence of fluorophores with characteristic excitation/emission bands at 550-570/600-650 nm in the PDO Vinagre de Jerez, and at 530-605/585-655 nm ex/em in the PDO Vinagre de Montilla-Moriles, related to the presence of sugars, grape-must caramel or furfurals.

1.5. In addition, EFM, in combination with chemometric techniques, demonstrated the ability to detect and quantify for the first time and quickly and economically, the amount of must added caramel to a wine vinegar, with a high correlation coefficient (R2> 0.921) and high prediction accuracy.

1.6. Spanish PDO wine vinegars, and their categories, were analysed for the first time by ^{1}H-NMR, obtaining a fingerprint useful to classify wine vinegars according to their PDO, with percentages of correct classification greater than 75%. The regions of the spectrum around 3.22-5.26 ppm, related to the presence of sugars, were relevant for the Pedro Ximénez category, while the region around 0.95 to 2.5 ppm was highlighted for the less aging categories, related to the presence of acetic acid and acetoin.

1.7. The mid-level data fusion and a data fusion based on the predictive analysis of common and specific multi-block weights (P-ComDim) have been compared, for the first time, using the spectroscopic data obtained by the aforementioned techniques, and using them for first time in two types of matrices, 2D and 3D. In addition, P-ComDim, a very recently proposed method, has been applied for the first time in a 3D data matrix. This provided a more complete spectroscopic characterization of the Spanish PDO wine vinegars, in addition to a correct classification of 90-100% of the samples according to the PDO, regardless of the category to which they belonged.

1.8. The UV-vis spectra of the wine vinegars analysed showed observable differences at first sight and responsible for their correct classification: wines vinegar from the PDO Vinagre de Jerez of the aged categories were characterized by UV-vis bands of greater intensity than the other PDOs, with maximums at 310 nm (the least aged) and 330 and 360 nm (the oldest), while the aged categories of the PDO Vinagre de Montilla-Moriles showed the lowest intensity in the absorption bands in the UV range -vis compared to the other two PDOs, with maximums at 295 nm (the least aged) and 300 nm (the oldest). In addition, the wine vinegars from the PDO Vinagre de Condado de Huelva presented UV-vis bands at intermediate intensities between the other two PDOs, with maximums at 350 nm (the least aged) and at 330 nm (the oldest), in addition to at 290 nm and the lowest intensity observed for the non-aged category.

1.9. The analysis of the samples by UV-vis spectroscopy provided the best classification models (100% of correct classification of all the samples), providing a hierarchical classification model capable of differentiating: the method of production of a wine vinegar, traditional or fast or, what is the same, vinegars with PDO from vinegars without PDO; the aging time or categories; wine vinegars of different PDOs; as well as the different categories within each PDO.

1.10. The stable isotope analysis of carbon ($\delta^{13}C$) made possible to indicate if the acetic acid and the sugars present in the wine vinegars studied were from the grape, while the stable isotope analysis of oxygen ($\delta^{18}O$) allowed to discriminate all the Spanish vinegars according to

the three geographical coordinates (latitude, longitude and altitude), with positive values associated with vinegars from the south (i.e. wine vinegars with PDO) and negative values with vinegars from the north (i.e. wine vinegars without PDO). In addition, different $\delta^{18}O$ values were observed among the 3 PDOs, being the lowest values observed for the PDO Vinagre de Montilla-Moriles (1.91 ± 1.11 ‰), intermediate for the PDO Vinagre de Jerez (1.91 ± 1.11 ‰) and higher for the PDO Vinagre de Condado de Huelva (2.67 ± 1.45 ‰).

1.11. The classification results obtained and the methodologies studied enabled developing a classificatory tool for wine vinegars, named as "VinegarScan", implementing the hierarchical classification model obtained with UV-vis spectroscopy data. This method has the advantage of having been carried out by means of a portable equipment that allows quick, simple and economic analyses. Therefore, this tool, currently registered in the Intellectual Property Registry of the US, could be implemented in wineries or wine vinegar control agencies.

2. Regarding the results obtained by the aromatic characterization of Spanish PDO wine vinegars and their commercialized categories by HSSE-GC-MS, GC-MS-O and sensory analysis, the following conclusions are drawn:

2.1. After the evaluation of three extraction techniques for the analysis by GC-MS of wine vinegars with PDO, the headspace sorptive extraction (HSSE) technique was selected because it extracted a greater number of compounds, allowed a better differentiation of the PDOs and categories, and had more advantages than other techniques studied.

2.2. The volatile profile obtained by HSSE-GC-MS of the wine vinegars of each PDO and of each of their categories was different and characteristic, allowing to correctly classify the PDOs, as well as to select certain volatile compounds as markers, that could be responsible for this differentiation. Among them, it could be highlighted trans-2-hexenyl, trans-2-hexen-1-ol and 5-hydroxymethylfurfural (5-HMF) for vinegars of the PDO Vinagre de Jerez; diacetyl, acetoin, and 2- and 3-heptanone for the PDO Vinagre de Montilla-Moriles, and 1-heptanol, methyl nonanoate, eucalyptol and α-terpineol for the PDO Vinagre de Condado de Huelva.

2.3. The aromatic characterization of the samples by means of GC-MS-O enabled differentiating the three PDOs by means of a series of aromatic markers, called impact odorants, selected according to the results of modified frequency (MF) and the values of aromatic activity (OAV). Thus, the impact odorants for the Reserva vinegars of the PDO Vinagre de Jerez were ethyl propionate, ethyl octanoate, propanoic acid, phenethyl acetate and 4-ethylphenol, while diacetyl, methional and furfural for the Pedro Ximénez category. With respect to the Reserva category of the PDO Vinagre de Montilla-Moriles, the selected impact odorants were acetoin and abhexone, while for the Pedro Ximénez were phenylethyl acetate and vanillin. The impact

odorants for the Reserva category of the PDO Vinagre de Condado de Huelva according to FM and OAV were acetaldehyde diethylacetal, isobutyl acetate, ethyl isovalerate and guaiacol.

2.4. The aromatic characterization by GC-MS-O and by sensory analysis demonstrated the sensory differences and aromatic notes characteristic of each of these vinegars. The vinegars from the PDO Vinagre de Jerez showed a higher percentage of impact odorants responsible for green and herbaceous nuances, and sweet, spicy and liqueur notes for its Pedro Ximénez category; the vinegars of the PDO Vinagre de Montilla-Moriles showed a higher percentage of impact odorants responsible for a butter-lactic-cheese aroma, and sweet, toasted and spicy nuances for its Pedro Ximénez category; and vinegars from the PDO Vinagre de Condado de Huelva showed a higher percentage of impact odorants responsible for chemical, pungent and fruity nuances.

3. The knowledge generated through the characterization of each of the Spanish PDOs of wine vinegar has shown the differential quality of these wine vinegars with PDO with respect to other vinegars, as well as the variety of qualities within PDOs. The differences observed are notable enough to develop control methods that enable authentifying and verifying the categories established within each PDO.

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8. ANEXOS

ANNEXES

ANEXO I

Vinegar

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FoodIntegrity Handbook: A GUIDE TO FOOD AUTHENTICITY ISSUES AND ANALYTICAL SOLUTIONS.

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Vinegar

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General overview of the product

Vinegar is one of the oldest fermented products in the world and its production dates back to around 2000 BC. Its acidic character (until the description of sulfuric acid, it was the strongest known acid) facilitated its use as a preservative due to its antimicrobial activity. Nowadays it is extensively used as a preservative, flavouring agent, and in some countries even as a healthy drink. Although, vinegar is mostly consumed in the food and beverage industry, it also finds applications in the healthcare and cleaning industry. The global vinegar market has reached values worth around USD 1.26 billion in 2017 growing at a rate of 2.1 % during 2010-2017 [1] and is further expected to reach a value of around USD 1.50 Billion by 2022.

As for fermented foods and beverages in general, the consumption of vinegar is a cultural trait. In Mediterranean countries, most vinegar is used directly or added to salads or to raw or cooked vegetables; thus, the appreciation of the organoleptic characteristics is straightforward. Therefore, "quality" vinegars are closely associated with these patterns of consumption. In contrast, in other countries, most vinegar is used for pickling or as part of sauces, and the impact of the organoleptic qualities, although possibly relevant for the final product, is less evident [2].

Types and major regions segment the global vinegar market. Different types of vinegar available are mostly balsamic vinegar, wine vinegar, cider vinegar, malt vinegar and rice vinegar. Geographically, Europe represents the biggest market for vinegar (more than half of the total global market share) followed by North America and the Asia Pacific region. In 2016, balsamic vinegar exhibited a clear dominance with the majority of market share. The use of vinegar is increasing in different cuisines, which results in increasing demand. Growing populations, rising disposable incomes, increasing health consciousness among consumers and the food and beverage

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industry are the main driving factors of the vinegar market. It is expected that the global vinegar market will witness growth both in terms of revenue and volume during the following years. Growth will come from changing consumer lifestyles and preferences. The interest in cooking gourmet and ethnic foods have increased among many consumers, thus prompting the sales of various dressings, most of which use vinegar as one of the key ingredients.

Some premium vinegars are being commercialised worldwide. A typical example of this trend is the increased consumption and trade of Balsamic Vinegar of Modena (Aceto Balsamico di Modena). In fact, Italy is the country that exports the most vinegar, providing twice the quantities of the other main exporters, Germany, Spain and France. Moreover, in terms of revenues, Italian vinegars are exported at much higher values than Spanish or German vinegars. The situation in Germany is different, considering that most German vinegar is sold for the pickling or sauce industry, whereas Spanish exports include also some premium vinegars such as Sherry vinegars (Vinagre de Jerez).

Sherry vinegars that are included in the European Union's Protected Designation of Origin (PDO) framework derive from Sherry wines and are necessarily aged in wood barrels for at least six months. This aging can be performed by a dynamic system (i.e., passage through different barrels containing vinegar from different vintages or different ages) or a static system. A more complex example is Aceto Balsamico, which is either Aceto Balsamico Tradizionale (ABT), regulated by two different PDO labels (ABT di Modena or ABT di Reggio Emilia), and Aceto Balsamico di Modena, which has a Protected Geographical Indication (PGI) status. The production of ABT is a long process that starts with the cooking of the grape must, which increases the sugar concentration to 400-500 g/L. Next, a partial alcoholic fermentation, which is initiated by osmophilic yeasts, produces a "sweet wine" of approximately 7 % (v/v) ethanol concentration and over 200 g/L of residual sugars. Then, some mother of vinegar is added to this "sweet wine," and it is left to be acetified. Once is acetified, the vinegar is placed in a "bateria" formed by five to seven barrels of different woods (oak, mulberry, chestnut, cherry, juniper, ash and acacia) and decreasing volumes (from 60 to 15 L), which are filled up to 2/3 of their total volume. This "bateria" is kept for at least 12 years with a yearly refilling from the previous barrel in a dynamic aging process. During this aging process, two phenomena occur: the transfer of components from the wood to the ABT and, more importantly, the concentration of the product and the integration of its components. The final product can have up to 500 g of sugar per kg of product, 7 % acetic acid (v/v) and 20 g of gluconic acid per kg. The oxidation of glucose by acetic acid bacteria yields gluconic acid. The result is a dark, concentrated and thick product sold in 100 mL bottles and with a market value that can easily reach 100 euros [3,4]. In contrast, Aceto Balsamico di Modena (ABM) is a PGI (Protected Geographical Indication) salad dressing ingredient now renowned throughout the world, obtained from cooked and/or concentrated grape must (at least 20 % of the volume), with the addition of at least 10 % of wine vinegar and a maximum 2 % of caramel for colour stability that is aged at least two months, not necessarily in barrels [5]. The geographical origin of ABM ingredients is not specified. However, some of these ABM can be aged for more than three years and are labelled "Invecchiato" (Aged). Overall, ABM is a cheaper version of ABT that has been popularized all over the world.

Some Asian vinegars, such as black vinegars from China or "kurosu" from Japan, are produced from rice and other cereals (including sorghum, wheat, and others) with a very important aging process in which concentration and thickening occur in a similar manner to ABT.

1. Product Identity

1.1. Definition of the product and manufacturing process

In general, food regulations consider vinegar the result of a double fermentation (alcoholic and acetous or acetification) of any sugar substrate, usually agricultural raw materials of plant origin with the exception of those produced from whey or honey.

In the European Union, the established limits for acidity and residual ethanol content are strictly set. Thus, the acidity of wine vinegar (acetification obtained exclusively from wine) must be at least 6 % (w/v), and the maximum residual ethanol allowed is 1.5 % (v/v) [4]. However, the variety of raw materials used in the production of vinegar is important, ranging from by-products and agricultural surpluses to high-quality substrates for the most unique and prized vinegars, such as Sherry vinegar (Spain) and Aceto Balsamico Tradizionale (Italy). There are up to ten types of vinegars, which include wine, fruit, cider, alcoholic, cereal, malt, malt distillate, honey and whey vinegars. Undoubtedly, wine vinegar is the most common type in Mediterranean countries, although the latest gastronomic trends have led to a considerable expansion of the varieties available in recent years. However, worldwide most of the vinegar produced is “white” vinegar, that is, vinegar produced directly from diluted alcohol [3]. In Asia, rice vinegar is the most common type, although other types are also found, many of them following very traditional systems of production.

Table 1: Different vinegars of the world are classified according to substrate, name and region/country of production and distribution [6]

Substrate (Raw material)	Name	Region/Country (Production & distribution)
Grape	Wine vinegar	Global
	Balsamic vinegar	Global
	Red vinegar	Global
	White vinegar	Global
	Distilled white vinegar	Global
	Sherry vinegar	Global
	Traditional Balsamic vinegar	Global
Apple	Cider vinegar	US, Canada
Different fruits (mango, kaki, berries)	Fruit vinegar	East and Southeast Asia
Date	Date vinegar	Middle East
Coconut	Coconut vinegar	Tropical Africa
Rice	Rice vinegar	China, Japan, Korea
	Kurosu	China, Japan, Korea
Malt	Malt vinegar	USA, Northern Europe
	Distilled malt vinegar	USA, Northern Europe
Whey (dairy by-products)	Whey vinegar	Europe
Honey	Honey vinegar	Global

Therefore, vinegars can be classified by their substrates of origin or by their systems of production. It is necessary to differentiate between those products derived from the double fermentation of a single fruit (most often grapes or apples) and those that are “flavored” vinegars, that is, vinegars of various origin with added concentrates of different fruits, flowers, or spices. Although these “flavoured” vinegars are not considered vinegars in some countries, lax regulations in other countries allow these products or condiments to be sold as “vinegars”.

The first fermentation is an alcoholic fermentation and transforms sugars or processed starches into ethanol. This process is performed by yeast, mostly from the species *Saccharomyces cerevisiae*, although some other species can also perform the alcoholic fermentation, partially or totally. The final result is considered the substrate of the second transformation, to convert ethanol to acetic acid. Although this second process is often referred as “acetic” or “oxidative” fermentation, it is not biochemically a fermentation but an oxidation. The proper term is thus “acetification” and involves a two-step oxidation, from ethanol into acetaldehyde and further from acetaldehyde into acetic acid, whereby the end of this process requires an electron acceptor, with molecular oxygen being the most common [2]. The microorganisms responsible for this process are acetic acid bacteria. These bacteria are found on substrates containing sugars and/or alcohol, such as fruit juice, wine, cider and beer. On these substrates, the sugars and alcohols are incompletely oxidized, leading to the accumulation of organic acids, such as the production of acetic acid from ethanol. Although more than 60 species have been described as acetic acid bacteria, only a limited number of them are involved in the production of vinegar. The species most commonly found in the production of vinegar are *Acetobacter pasteurianus, Komagataei bactereuropaeus* and *Komagataei bacterxylinus*. Several attempts have been done to have single, well-defined species of acetic acid bacteria for the production of vinegar, although it has been concluded that a mixture of at least two species (one of them as “starter” and the other as “finisher”, with different acetic acid sensitivities) is the most appropriate to be used as inoculum for the production of vinegar, especially those above 5 % (w/v) acetic acid [7,8].

Vinegars can also be differentiated by their production systems. In traditional vinegars, the transformation of ethanol into acetic acid is performed by a static culture of acetic acid bacteria at the interface between the liquid and air. Barrels are filled to 2/3 of their capacity, as to leave an air chamber, which is kept in contact with the outside air using one opening or various types of openings. This production system, which is considered to be the traditional method, is called “surface culture”. A more standardized version of this method, the “Orleans method,” includes side holes for air circulation and adds a funnel with an extension to the base of the barrel to allow wine to be added at the bottom of the barrel, preventing the alteration of the "mother of vinegar". This mother of vinegar is the biofilm formed by the transforming microorganisms, i.e. the acetic acid bacteria, which develops on the surface due to the need for oxygen. The vinegars produced by this traditional system are generally considered of high quality because of their organoleptic complexity, which is mainly due to the metabolism of the acetic acid bacteria and the overlapping vinegar production with aging. However, this process is very slow, and the production of vinegar can take from months to years.

To reduce the acetification time, other methods, such as the Schutzenbach systems with submerged cultures, have been developed. Bacteria are immobilized on wood chips or charcoal, forming a solid bed on which the vinegar spreads. After passing through the bed of chips, the vinegar is collected in a container at the bottom and pumped back to the same fixed bed. The acidity successively increases, and it is possible to obtain vinegar of reasonable quality within a week.

Submerged culture systems provide a much faster alternative. These systems rely on suitable turbines to generate a flow of air bubbles into the wine or alcoholic solution. The oxidative process occurs in the air-liquid interfaces of the air bubbles. Improvements to this process generally involve engineering (improving the maintenance and persistence of the bubbles in the liquid, the uniformity of the bubble size, the recovery of lost aromas, etc.). Vinegar is then produced at a significantly lower cost, the bacteria act as bioreactors for the transformation of alcohol into acetic acid, the airflow contributes to a considerable loss of the volatile compounds present in the original alcoholic solution, resulting in a less complex product from a sensory point of view. Although early containers for submerged culture processing were made of wood, the usual containers are stainless steel, which is more hygienic and resistant to acidic conditions. The limitations can be compensated by subsequent aging in barrels or by submerging wood fragments or wood chips, which may contribute to the recovery of some of the missing organoleptic character. Despite the loss in product quality, this methodology has two important advantages: speed (the vinegar can be produced in cycles of 24 hours, or even shorter) and acidity (the product can reach concentrations of acetic acid of up to 23-25 %, compared to the 6-13 % achieved with other systems). Higher acidity helps to reduce transportation costs by reducing water transport.

An important aspect that contributes to the organoleptic quality of vinegars is aging, which enables the integration of the different compounds in vinegars. The increase in organoleptic quality during aging is remarkable; in addition to interactions with the wood, a series of chemical reactions, evaporation, the production of esters, reactions between acids and residual alcohols, and other processes result in better integration of aromas and metabolites and a reduction in the pungency of acetic acid.

1.2. Current standards of identity or related legislation

Vinegar is regulated by different standards, and even the legal definition itself varies from country to country [3]. The regional European Codex standard for vinegar dates back to 1987 [9], and it states that vinegar is as any liquid fit for human consumption, produced exclusively from suitable products containing starch and/or sugars by the process of double fermentation, first alcoholic and then acetous. Although several attempts have been made to convert the regional standard into a world-wide standard, this conversion has so far not been addressed, especially in view of trade patterns and significant regional differences. The standard describes different kinds of vinegar, essential composition and quality criteria together with optional ingredients, contaminants, hygiene, weights and measures as well as methods of analysis. This regional standard has not taken up by all national legislations of the Member States due to the fact that in two States the name ‘vinegar’ applies to the product obtained by dilution of synthetic acetic acid.

In the USA, the FDA (Food and Drug Administration) requires that vinegar products must contain at least 4 % acids. There are no FDA standards of identity for vinegar, however the “Compliance Policy Guides” establishes the labelling requirements for cider, wine, malt, sugar, sugar and vinegar blends.

In the EU, Regulation (EC) 1493/1999 [10], there are currently established thresholds for acidity and residual alcohol. Hence vinegars are those products having a minimum 5 % (w/v) acidity and a maximum of 0.5 % (v/v) ethanol, with the exception of wine vinegar which is exclusively obtained from wine and whose acidity is 6 % /w/v) at least and has a maximum ethanol concentration of 1.5 % (v/v). More recently the European Commission published Commission Regulation (EU) 2016/263 [11] amending Annex II to Regulation (EC) No 1333/2008 [12] of the European Parliament and

Council as regards the title of the food category 12.3 Vinegars. The new title of the food category 12.3 is now: Vinegars and diluted acetic acid (diluted with water to 4-30 % by volume)**.** This category was renamed because *in some Member States only vinegars obtained from the fermentation of agricultural products are allowed to be named 'vinegars'. In other Member States, however, both products obtained from the dilution with water of acetic acid and vinegars obtained from the fermentation of agricultural products are marketed under the name 'vinegar'.*

Three EU schemes of geographical indications and traditional specialties, known as Protected Designation of Origin (PDO), Protected Geographical Indication (PGI), and Traditional Specialities Guaranteed (TSG), promote and protect names of quality agricultural products and foodstuffs. Products registered under one of the three schemes may be marked with the logo for that scheme to help identify those products. The schemes are based on the legal framework provided by EU Regulation No 1151/2012 [13] of the European Parliament and of the Council of 21 November 2012 on quality schemes for agricultural products and foodstuffs. This regulation (enforced within the EU and being gradually expanded internationally via bilateral agreements between the EU and non-EU countries) ensures that only products that originate from that particular region are allowed to be marketed as such. Regarding vinegars, there are currently five PDO registered categories and one PGI. Among PDOs: three from Spain (Vinagre de Jerez, Vinagre de Montilla-Moriles, Vinagre de El Condado de Huelva) and two from Italy (Aceto Balsamico Tradizionale di Modena, Aceto Balsamico Tradizionale di Reggio Emilia). Lastly Aceto Balsamico di Modena is registered as a PGI.

Currently there is no European trade association of vinegar producers. The Vinegar Institute is the international trade association representing the vast majority of vinegar manufacturers and bottlers, mainly those with activities in the USA.

2. Authenticity issues

2.1. Identification of current authenticity issues

2.1.1. Framework of national and international legislation

Due to the observed differences in the laws on vinegar from one country to another, it is clear that if a vinegar produced in one country is commercialized in another in which the definition of vinegar changes, it poses a problem and risk for consumers and can become an authenticity issue if its origin is not clearly declared. Thus, a number of examples exist where a vinegar from one country is commercialized in other country in which the legal definition of this kind of vinegar varies. For example, while in the European Union, the term vinegar describes 'a product of a double fermentation (alcoholic and acetic fermentation) from substances of agricultural origin', in the USA a 'synthetically-produced acetic acid diluted with water' can also be labelled as vinegar. Hence, if the latter is sold in Spain, it could be considered a fraud to the consumers. Other example of this problem occurs between Germany and Europe. The German legal definition of 'wine vinegar' permit the production of vinegar by acetic fermentation from natural ethanol, by diluting acetic acid with water or by blending fermentation vinegar with synthetic acetic acid, or with vinegar made from synthetic acetic acid [14]. However, European regulations indicate that wine vinegar can only be produced through the acetic fermentation of wine produced from fresh grapes. So commercialising some 'wine vinegars' from Germany produced with alcohol from different origins as genuine wine vinegar in a European country, could mislead the consumer.

2.1.2. Raw materials

One of the main problems in the vinegar industry lies in the difficult distinction between the use of low-quality and high-quality raw materials, between true vinegars rich in extracts from the raw materials or their blends, as well as to distinguish between highly valued, high quality wine vinegars or aged balsamic vinegars and their cheaper alternatives derived from other raw materials such as malt or alcohol and/or vinegar adulteration with diluted synthetic acid [15]. Within this section, the following issues are discussed.

2.1.2.1. Addition of chemical acetic acid

One of the first frauds in the vinegar industry, and one that has been occurring for more than eighty years, is the addition of chemical or non-biological acetic acid to different types of vinegar contrary to the vinegar industry regulations. The vinegar obtained by chemical acetic acid is called *wood vinegar* or *vinegar essence*, and it cannot be sold as *fermented vinegar* due to it contains more heavy metals per kg of pure acetic acid that the regulated permitted amount (maximum of 5 mg/kg pure acetic acid), which supposes a risk for the consumer. In this sense, European legislation indicates that authentic wine vinegar cannot contain acetic acid obtained from either petroleum derivatives or wood pyrolysis (synthetic acetic acid). These adulterated products constitute a fraud for consumers and are unfair practices to other vinegar producers. To detect the addition of chemical acetic acid to vinegar, the determination of formic acid, derived from the pyrolysis of wood, has demonstrated to be an indirect indicator of it [16], although the detection of synthetic acid added to spirit vinegar or to relevant products produced with the adulterated vinegar or synthetic acetic acid still remains difficult.

2.1.2.2. Addition of water to dried grapes or to must concentrate

The production of vinegar from dried grapes diluted with water is an unfair practice more related to the industry of wine vinegars. This so-called 'raisin vinegar' is commonly produced in some Mediterranean countries by fermenting dried grapes and rehydrating with tap water, but it cannot be regarded, or labelled, as 'wine vinegar'. Due to the fact that this method reduces the price of production, it can be considered, in some Europe countries, as a fraudulent activity. Thus, it has been noticed that some Greek vinegars produced by the above water addition method have been improperly imported into Italy as 'wine vinegar' [17].

2.1.2.3. Use of alcohol or sugar not from wine

Commercialising vinegars produced with alcohol from different origins other than grapes, as genuine wine vinegar, is one of the most common fraudulent activities in the vinegar industry. This fraudulent practice aims to reduce manufacturing costs and constitutes a fraud to consumers. Another unfair practice that is currently happening, is the addition of different proportions of alcohol vinegar to wine vinegar samples, which makes the product cheaper. This unfair economic advantage poses an important threat for this sector. These adulterations are difficult to detect because the alcohol added to the base wine prior to the commencement of the fermentation process does not always have a well-known botanical origin [18]. The alcohol added to wine vinegars should come from the fermentation of skins of grapes, but sometimes its origin is fairly diverse: molasses, sugar beet, or sugar cane. Therefore, authenticity issues arise in the ability to detect if the source of the acetic acid and the grape sugars is truly grape (wine) ethanol or wine must, or other ethanol made from fermentation of some other cheaper agricultural products (cereal, potato starch, beetroot or sugarcane), that is called synthetic acetic acid. In the case of

balsamic vinegar as Aceto Balsamico di Modena IGP, there could also be the unfair practice of adding exogenous sugars to cooked and/or concentrated grape must.

2.1.2.4. Blends of different type of vinegars

Another common fraudulent practice in the elaboration and commercialisation of vinegar is the mixture of different proportions of wine vinegar and alcohol vinegar. The authenticity issue in this case occurs when this blend is sold under the denomination of wine vinegar, as if it was a pure product. Generally, a good method for a safe differentiation between them is the identification of specific fruit acids, although this can be manipulated easily with the addition of fruit-specific acids and amino acids.

2.1.3. Authentication of geographical indications

The existence of protected origin designations or quality labels in vinegars, which is very common in Southern Europe, provides a greater guarantee to the product although, at the same time, encourages the picaresque nature of unfair producers. The basic requirements for the product to receive such protection is that it must be closely associated with a particular geographical area and with a traditional production procedure which account for the specific quality and characteristics of the vinegar, and therefore, they have higher prices. Some of these characteristics that are defined and established under the PDO Regulations and are mandatory for these vinegars are for example, total acidity, total dry extract or total ash content. Although these PDOs strictly regulate these parameters - all regularly controlled by an inspection authority - some adulteration or frauds have occurred. All too often, however, they are condoned by leading manufacturers, mainly due to the powerful argument of extra profit. Examples include the well-known case of Traditional Balsamic Vinegar of Modena PDO (Protected Designation of Origin) and the Balsamic Vinegar of Modena PGI (Protected Geographical Indication). The former is produced by a traditional, time-consuming and expensive production method obeying very strict rules of raw material provenance and production methods, ensuring a high quality. The second one is produced industrially and is a much cheaper product made from cooked must, concentrated must and wine vinegar via a complicated process [19,20]. Due to their different prices, frauds and mislabelling are frequent, and many brands of these popular vinegars commercialised in the market are in fact merely a sweetened red wine vinegar with food colouring.

Also of considerable interest is the differentiation between Spanish PDO vinegars. Good and promising results in the characterisation and classification of these PDO vinegars have been achieved using different analytical procedures [21-24], but there is still a long way to go. The need to develop methods to distinguish vinegars with this recognised label from non-authentic product is obvious, as not only will the consumer be cheated, but he or she will lose confidence in PDO/IGP labels.

2.1.4. Production process and aging

Adulteration related to production processes occur mainly in vinegars produced by traditional systems such as Sherry vinegar or Traditional Balsamic Vinegar of Modena and Reggio-Emilia. There is an increased interest in differentiating vinegars that have been produced by a traditional method from those produced by a quick production method, due to the fact that the former is associated with a higher quality but also with a longer processing time and a higher cost of production. A further authenticity issue arises when there is a specified minimum aging time for a particular vinegar, as in the case of Sherry vinegars or Traditional Balsamic Vinegar of Modena, the latter being only sold after following an ageing process of at least 12 years in a set of wooden casks

of decreasing volume [19]. The organoleptic vinegar properties developed during ageing make the finished product very appealing. Nevertheless, the production time and costs are too excessive to permit a lucrative trade. Hence, an objective of the vinegar industry is to produce these aged vinegars with the same organic characteristics related to aging, but making it in the most economic and rapid way. For these reasons, the vinegar industry has a very real interest in speeding up ageing if this can be done in a way which does not produce an inferior product or result in the consumer being misled. In this context, the use of wood chips is being investigated. Moreover, there is an increasing necessity to develop simple methods able to detect specific metabolites in vinegars as possible indicators for the ageing process and traditional procedures, in order to protect the consumers and avoid unfair competitions.

2.1.5. Adulteration by addition of grape must caramel

The colour of the vinegars is an important quality parameter as it can, for example, indicate that a wine vinegar has undergone a process of aging in wood barrels. The wine vinegar colour changes during aging from amber to mahogany due to the changes that occur, in the content of polyphenols, tannins and anthocyanins as well as an oxidation process, which are responsible for the darkening of the vinegar. In this context, although the addition of grape-must caramel is allowed by the current legislation to correct and unify the final colour of the different batches, sometimes it could be added to simulate the effect of a greater aging of wine vinegar in wood, which would be considered as an unfair practice.

3. Analytical methods used to test for authenticity

3.1. Officially recognised methods

To assess the quality and authenticity of vinegars, several countries have established acceptable methods and ranges or guide values for some vinegar parameters, based on results obtained on the analysis of a large numbers of authentic samples. However, current national and international directives include more methods designed for vinegar identification and generally control than for authenticity issues. In this section officially recognised methods used on a regular basis for vinegars are described (cf. Table 2).

Table 2: Officially recognised methods to test for vinegar authenticity

Method	Reference	Technique	Objective
For wine vinegars			
Determination of total acidity content	OENO 52/2000	Neutralisation of acids in sample by alkali solution	To comply with legal requirements (definitions, PDO, PGI…)
Determination of the fixed acidity content	OENO 53/2000	Neutralisation of the (non-volatile) acids of the residue in an aqueous solution using an alkali solution	To comply with legal requirements (definitions, PDO, PGI…)
Determination of the volatile acid content	OENO 54/2000	Calculation of difference between total acidity and fixed acidity, expressed in grams of acetic acid per L	To comply with legal requirements (definitions, PDO, PGI…)
Detection and quantification of the presence of synthetic acetic acid	OENO 55/2000	After extracting the acetic acid using sodium hydroxide, complete by liquid scintillation the reactivity ^{14}C of the product converted into benzene	Authentication: Values less than the characteristic ^{14}C contents of the assumed year of production represent either a mixture with products from more recent years, or the addition of all or part of the synthetic acetic acid
Determination of the residual alcohol content	OENO 56/2000	Distillation of vinegar, oxidisation of ethanol by potassium dichromate and determination of its content by titrating the excess potassium dichromate by a solution of iron sulphate and ammonium	To comply with law requirements (legal definitions, PDO, PGI…)
Determination of total dry extract content	OENO 57/2000	Evaporation of sample and drying in oven, then weighing	Detection of frauds: the addition of water or an aqueous solution of acetic acid (very low total dry extract value) or the addition of non-volatile substances (very high total dry extract value). Database for the type and origin of the vinegar is necessary.
Determination of ash content	OENO 58/2000	Incineration of the vinegar extract between 500°C and 550°C through to complete combustion of the carbon	Detection of frauds: the addition of water or an acetic acid aqueous solution (very low ash content) or the addition of non-volatile substances (very high ash content). Database for the type and origin of the vinegar is necessary.
Determination of the non-volatile reducing substances content	OENO 59/2000	Evaporation of volatile substances, hydrochloric hydrolysis, oxidisation by a copper alkali solution in excess with titling by iodometry of copper ions	Detection of frauds: the addition of non-volatile substances.
Determination of the total sulphur dioxide content	OENO 60/2000 + OENO 13/2008	Iodometric titration direct (free SO_2) and after double alkaline hydrolysis (combined SO_2)	Control the level of SO_2 and check compliance with standards
Determination of the total ascorbic acid content	OENO 61/2000	Oxidisation of ascorbic acid by iodine with transformation into dehydroascorbic acid, precipitation with 2.4 – dinitrophenylhydrazine. Separation by thin film chromatography, solubilisation in acetic medium and colorimetric determination at 500 nm.	Detection of a fraudulent technological use.

Method	Reference	Technique	Objective
Measurement of chloride content	OENO 62/2000	Potentiometric titration of Cl ions with a solution of silver nitrate, in an acidic environment, after prior measurement of the potential equivalent point of a standard chloride solution	Detection of the fraudulent increase in the dry extract by the addition of sodium chloride
Measurement of sulphate content	OENO 63/2000	Precipitation of sulphates with barium chloride, drying, calcination and weighing	Detection of frauds (aimed at increasing the total dry extract).
Measurement of copper content	OENO 64/2000	Direct measurement by atomic absorption spectrophotometry.	Contamination from contact materials during manufacture, and the iron of the wine itself. Excessive content could cause haze or serious alterations in colour.
Measurement of zinc content	OENO 65/2000	Direct measurement by atomic absorption spectrophotometry.	Contamination from contact materials during manufacture, and excessive content could cause hazes or serious alterations in colour.
Measurement of iron content	OENO 66/2000	Direct measurement by atomic absorption spectrophotometry.	Contaminations from contact materials during their manufacture, and of course the iron of the wine itself. Excessive content could cause haze or serious alterations in the colour.
Measurement of lead content	OENO 67/2000	Direct measurement of lead content in the vinegar by atomic absorption spectrometry without flame (electrothermal atomisation).	The presence of lead in vinegars mainly has its origin in contaminations from contact materials during their manufacture, and the lead of the wine itself from which the vinegar has been made
Measurement of mercury content	OENO 68/2000	Mineralisation. Reduction by permanganate Measurement by atomic absorption spectrometry (cold vapour).	Toxicologic issue
Measurement of the acetoin content	OENO 69/2000	Neutralisation of the sample at pH 7.00 with calcium hydroxide. Direct measurement of the acetoin via gas chromatography	Authentication: Determination of quality and origin by the analysis of acetoin content in the wine vinegars (between 100 mg/L and over 400 mg/L)
Measurement of the methanol, superior alcohols and ethyl acetate	OENO 70/2000	Neutralization of the sample at pH 7.00 with a sodium hydroxide solution. Measurement, via GC, of some volatile components: methanol, propan-1-ol, butan-2-ol, 2-methylpropan-1-ol, butan-1-ol and 2-methylbutan-1-ol + 3-methylbutan-1-ol	Organoleptic and possibly toxicologic issue
Authentication by SNIF-NMR® and other isotopic methods	OENO 71/2000	Extraction of the acetic acid from the vinegar with ether. Purification using a Cadiot column. Determination of the purity of acetic acid. Measurement of the site-specific deuterium/hydrogen ratio in the resulting acetic acid, via deuterium NMR.	Detection of frauds: detection of synthetic acetic acid in vinegars and any other downgrading of vinegars. Detection of possible addition of alcohol-vinegar coming from plants whose metabolism is C_4 (sugar addition from cane) or C_3 (beet)

Method	Reference	Technique	Objective
Detection of synthetic acetic acid in wine vinegars by the determination of beta radioactivity of ^{14}C of acetic acid by liquid scintillation	OENO 12/2006	Extraction of acetic acid from the vinegar. Acetic acid of mineral origin (Control) is counted in the same way. β emission value of the ^{14}C in the sample compared with the average value of the β emissions of ^{14}C found in the ethanol in genuine late harvest wines.	Detection of fraud: detection of the addition of synthetic acetic acid (levels lower than those for a given year) or the entire content of it. Control of the year of production of the raw wines.
Method for $^{13}C/^{12}C$ isotope ratio determination of acetic acid in wine vinegar by isotopic mass spectrometry	OIV-OENO 510-2013	$^{13}C/^{12}C$ isotope ratio of acetic acid by Isotope ratio mass spectrometry (IRMS)	Detection of frauds related to the botanical origin of acetic acid and revelation of the addition of synthetic acetic acid. Determination of sugar addition (cane)
Method for $^{18}O/^{16}O$ isotope ratio determination of water in wine vinegar using isotopic mass spectrometry	OIV-OENO 511-2013	$^{18}O/^{16}O$ isotopic ratio of water by Isotopic Ratio Mass Spectrometry (IRMS)	Detection of frauds related to the production of vinegars from fresh grapes or from dried grapes with water addition
Determination of the distribution of deuterium in the acetic acid of vinegar wine by Nuclear Magnetic Resonance (NMR)	OIV-OENO-527-2015	Composite ^{1}H-NMR and ^{2}H-SNIF–NMR	Detection of frauds about botanical origin of acetic acid and revelation of the addition of synthetic acetic acid
For all vinegars			
Isotopic analysis of acetic acid and water Part 1: ^{2}H-NMR analysis of acetic acid. Part 2: ^{13}C-IRMS analysis of acetic acid. Part 3: ^{18}O-IRMS analysis of water in wine vinegar	CEN, EN 16466-1,2,3 (2012)	SNIF-NMR (D/H), $^{13}C/^{12}C$ IRMS, $^{18}O/^{16}O$ IRMS	Determination of frauds related to vinegar acetic acid, water and sugar addition (beet, cane)

3.2. Other commonly used methods

3.2.1. Sensory analysis

Sensory analysis has proven to be a simple and reliable tool for assessing the quality of vinegars [25]. The appropriate sensory methodology must be clearly defined and the attributes used in discriminant or descriptive analysis must be precise and well-recognised by the panel. The sensory characterisation of vinegars for monitoring vinegar quality has been widely performed in many studies over a number of years [26-31]. Moreover, in some vinegars, quality control is mainly based on their sensory properties, as is the case for Traditional Balsamic Vinegar of Modena. Sensory vinegar analysis can be performed by olfactive and gustative analyses, as well as by the determination of other parameters such as viscosity and colour.

3.2.1.1. Odour and taste

In order to analyse the taste and odour of the vinegars, there are different protocols such as preparing the vinegar in a way that most resembles how it is normally consumed (using lettuce suspended in the vinegar [27]or diluting with cold or hot water), or testing and smelling vinegar as is, using opaque cups to avoid colour influences, being it the usual sensory analysis for vinegar cellars [26].

Within the different types of sensory analysis, the most used are the descriptive test, that is useful for determining the sensory profile of the samples, and the discriminatory test, which include a wide range of tests such as triangular test (ISO, 2004, standard 4120) [32] and Paired Comparison tests (ISO, 1983b, standard 5495) [33], preference test, etc. These methods require a well-trained testing panel, and concrete and adequate attributes.

3.2.1.2. Viscosity

Viscosity is another important parameter in the sensorial quality of some vinegars such as in the case of the Traditional Balsamic Vinegar of Modena. Nevertheless, no procedure has yet been established to determine this objectively, as it is assessed in an empirical manner and wrongly expressed as physical density.

3.2.1.3. Colour

Colour is one of the most important parameters used by consumers to assess the quality of a food product. Some studies have described a relationship between some compounds and a darker colour such as melanoidin, and products from the degradation of sugars and Maillard reactions [3]. A darker colour is also related to a longer aging period in wine vinegars and Traditional Balsamic vinegar of Modena. Some techniques such as UV-Visible spectrophotometry or excitation-emission fluorescence or transmission colorimetric techniques are being used with promising results for this issue [34-36]. However, the colour could be easily modified with the use of grape must caramel or other additives and no methods haves been officially established to assess and control this parameter.

3.2.2. Physicochemical analysis

Notwithstanding the fact that the quality of vinegars has been traditionally evaluated by using a trained sensory panel, more rapid and objective methodologies have been tested and performed by instrumental measurements.

3.2.2.1. Chromatographic techniques

Chromatographic techniques have been widely applied, for a long time, to determine certain vinegar compounds useful for characterising, classifying or detecting adulteration in vinegars.

High-performance liquid chromatography-mass spectrometry (HPLC)

HPLC has been widely used to analyse compounds such as phenols. Phenolic compounds are present in wine vinegars due to their natural content in grapes or as a result of contact with wood during the aging process, and they have demonstrated to be important in the determination of origin and the technology involved in the production of wine vinegars [37-39].

Gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS)

Gas chromatography (GC) is the official method for the determination of acetoin content, methanol, superior alcohols and ethyl acetate (OENO 69-70/2000) [40,41], and has also been applied to determine poly-alcohols in vinegars, all of them related to quality and origin. In addition to this method, gas chromatography coupled with mass spectrometry (GC-MS) has been the most efficient and widely employed technique to date to determine the volatile composition of vinegars which is also directly related to the quality of the vinegar. This technique normally requires a prior extraction step (such as dynamic and static headspace extraction, solid phase microextraction, stir bar sorptive extraction or liquid-liquid extraction methods). Examples of the efficiency of this

methodology are the determination of volatile aldehydes as discriminant parameters in quality vinegars or the determination of the volatile profile as a classification parameter of different vinegar types or geographical indicators [42-45]. However, regardless of the fact that these sampling methods have been widely employed in the volatile analysis of vinegars, the experimental sources of variability related to GC–MS (e.g. columns, stationary phase, temperature or experimental conditions and sample preparation) still cause some variations that directly affects the final results. These problems are being recently resolved by chemometric tools such as Multiple curve resolution (MCR) or Parallel factor analysis (PARAFAC) [44].

Gas chromatography coupled with olfactometry (GC-O)

The intensity and quality of the aroma constitutes the primary quality factor in vinegars. Although the aroma of vinegars is widely studied by sensory analysis and GC-MS methodologies, all volatile compounds determined in vinegar do not have the same contribution to the overall aroma of the product. Gas chromatography-olfactometry (GCO) is the most appropriate analytical technique to determine these compounds with real impact of the aroma of a vinegar, known as impact odorants, among the whole volatile fraction. This technique provides instrumental and sensory analysis simultaneously as the eluted analytes are perceived at the same time by the human nose and a conventional detector, such as the flame ionic detector (FID) or the mass spectra detector (MSD), which turns this technique into a powerful one in food aroma characterisation. However, little research can be found in the literature regarding the application of this technique in vinegars. Thus, only a few papers deal with a comprehensive characterisation of the aroma profile of red wine vinegars [31], some Chinese vinegars [46] or with the quality perception of Sherry vinegars [47].

3.2.2.2. Spectroscopic techniques

Near infrared spectroscopy

Near-infrared spectroscopy (in the range of 5000 - 15000 cm^{-1}) is a potential spectroscopic technique that has been applied to the analysis of vinegars. Near-infrared spectroscopy (NIR) has the advantages of high speed, accuracy, simplicity, and low cost. NIR spectra can record the multifrequency and co-frequency information of organic molecules, which involves the response of molecular bonds of C–H, N–H, C–O, and O–H, being useful for determining organic acids and pH in vinegars, as in the case of MIR, mentioned below [48]. The vinegar sample is either placed in a cuvette and the spectrum collected by absorption mode or the bottles can be directly scanned in transmission mode. A multivariate analysis of the data is usually employed to develop models able to classify the different classes of vinegars, different geographical origins [23,49] or even to predict or monitor the vinegar ageing process [50,51].

Mid-infrared spectroscopy

Mid-infrared spectroscopy (MIR) (in the range of 500 - 5000 cm^{-1}) has also been shown to be able to address a wide range of issues and provide solutions for rapid analysis and on-line control of vinegars. This technique combined with chemometrics has gained wide acceptance for authenticity and classification purposes in food, being informative at the molecular level and produces a single spectral fingerprint of each sample. Moreover, the use of an accessory of Attenuated Total Reflectance (ATR) allows the direct analysis of liquids in a simple, fast, only a few minutes, and non-destructive manner, involving minimal sample preparation. This method provides a greater amount of chemical information compared to NIR spectroscopy in terms of chemical assignment of observances and allows the interpretation of the spectra without the need of complex chemometrics. Thus, Fourier transform mid infrared spectroscopy (FT-IR) coupled with

ATR has been applied to investigate its potential as a tool for characterising different categories of high-quality vinegars by a studying the differences in the spectra. FT-IR spectra have also been used to predict the sensory score of traditional balsamic vinegar of Modena by the performance of different partial least squares (PLS) regression models [52] as well obtaining a full calibration model for organic acids in vinegars [53]. Finally, the technique can also be used to control certain steps and factors of the production processes in industry, making it possible to carry out necessary corrective actions without delay [54].

Fluorescence spectroscopy

Fluorescence spectroscopy is also being investigated as an alternative quality control tool for vinegars, with the same attributes as those mentioned above. Different methods of analysis are possible, the conventional one being the measurement of the excitation or emission spectra at a single emission or excitation wavelength, respectively. However, instead of measuring a single emission spectrum at a selected excitation wavelength, the emission spectra at different excitation wavelengths can be recorded, in a technique known as excitation-emission fluorescence. The latter results in a bi-dimensional Excitation- Emission Matrix (EEM), which contains unique information of each measured sample, having the advantage of containing more information about the fluorescent species than the conventional excitation and emission spectra separately. Moreover, the potential of the EEM technique can be improved by applying multivariate methods in the analysis of the fluorescence results such as Parallel Factor Analysis (PARAFAC) and its combination with PLS discriminant analysis. PARAFAC is used to decompose fluorescence EEMs into different independent groups of fluorescence components (fluorophores), as well as their relative concentration (scores) in each sample. This method extracts the most relevant information from the data in order to build further robust calibration and/or classification models. For this reason, this technique has been more widely applied in the study of wine vinegars than the simple excitation or emission analysis. Thus, Callejón et al. [48] and Ríos-Reina et al. [16] studied fluorescence excitation–emission spectroscopy combined with suitable multivariate methods. In these studies, the fluorescence Excitation-Emission Matrices (EEMs) were obtained by varying the excitation wavelength ranging between 250 and 700 nm (every 5 nm), and recording the emission spectra from 300 to 800 (every 2 nm). For these measurements, excitation and emission slits were both set at 5 nm, and the scan rate was fixed to 1200 nm min-1. These studies demonstrated this method's ability to characterise and classify three Spanish PDO wine vinegars according to their protected designation of origin, as well as their categories (aged and sweet) [24; 55]. However, despite the promising results obtained, is not yet widely in use in this field.

Nuclear Magnetic Resonance (NMR) Spectroscopy

NMR spectroscopy, which has the advantage of being a rapid and non-selective analysis without any manipulation or derivatisation, has recently achieved general acceptance as a powerful tool for vinegar quality and authenticity determination. NMR can provide information on chemical composition, concentration of soluble metabolites and their structure in the samples such as sugars, acids and flavonoids, with the advantage of providing the best combination of fast data acquisition and predictive capability. However, the large amount of data needs to be treated by multivariate methods such as principal component and discriminant analysis with the final objective of making models able to discriminate authentic and non-authentic vinegars, origins, or vinegar types.

Different nuclei to which the spectrometer is tuned have been investigated for vinegar authentication. The most commonly applied NMR technique for origin authentication, and recently recognised as an official method, is deuterium SNIF-NMR (Site-specific Natural Isotopic Fractionation studied by nuclear magnetic resonance spectrometry). However, another very used

method with promising results is proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy, which, combined with multivariate statistical data analysis, has demonstrated its usefulness in the characterisation of the ageing process and the discrimination of different vinegar types [19,56]. The application of ^{13}C NMR, two-dimensional ^{1}H–^{13}C heteronuclear multiple-bond correlation (HMBC), and ^{1}H–^{13}C heteronuclear single quantum coherence (HSQC) spectra for the characterisation and discrimination of Balsamic vinegars of Modena in order to obtain an indirect indicator of authenticity and a quality control tool have also been studied, although to a lesser extent [57]. It should be also considered that as vinegar samples contain a high amount of water, optimising water suppression methods is required, since it is one of the elements that most impacts the overall quality of the spectrum [58]. Moreover, as NMR generates a complex spectrum containing information on all proton/carbon bearing compounds, multivariate data analysis such as principal component analysis or discriminant analysis is employed to develop classification/authentication models.

3.2.2.3. Other techniques

Trace metal analysis

Trace metal analysis using inductively-coupled plasma optical-emission (ICP-OES), atomic absorption spectrometer spectroscopy (AAS), flame absorption (FAAS) and emission spectrometry (FES) has been applied to determine the mineral composition and the trace metal contents in vinegars to determine geographical origin, type of raw materials and different production processes [59,60]. Since the mineral composition of the plant reflects the mineral composition of the soil where it is growing, accordingly, soil differences and differences in grape varieties could be reflected in the mineral composition of the vinegars, providing information about the geographical origin. The main parameters found in the case of Spanish PDO wine vinegars were Ca, K, Mg, Na, P and S, that are natural components of grape juice, K being the pre- dominant cation.

Isotope analysis

The analysis of the isotope ratios of the bio-elements (^{2}H/^{1}H, ^{13}C/^{12}C, ^{18}O/^{16}O or ^{3}H/^{1}H, ^{14}C/^{12}C) has also shown to be useful for providing proof of vinegar authentication and for detecting frauds such as the addition of synthetic acetic acid or water and the source of this acid [22]. In fact, isotopic methods have been recently recognised by the European Committee for Standardization (CEN) and in part by the OIV as a means of detecting the presence of exogenous acetic acid and tap water in wine vinegars.

Recently [61] it was found that the above listed OIV and CEN methods for the analysis of stable isotope ratios D/H and ^{13}C/^{12}C in ethanol and acetic acid and of ^{18}O/^{16}O in water can be applied to the ingredients of balsamic vinegar such as Aceto Balsamico di Modena IGP to evaluate their authenticity. The standard deviation of repeatability and reproducibility are indeed comparable in wine vinegar and balsamic vinegar and generally lower than those quoted in the official methods. Moreover, no changes in the isotopic values from wine to vinegar and to balsamic vinegar, and from the original must to the balsamic vinegar must were found. This provide experimental evidence that reference data from isotopic wine databanks [61] can also be used to evaluate the authenticity of the ingredients of vinegar and balsamic vinegar.

4. Overview of methods for authenticity testing

The following table provides a summary of the methods and the authenticity issues they address.

Analytical Technique	Indicative data or Analyte	Authenticity issue or information
Colorimetric analysis	Total acidity content and fixed acidity content; total ascorbic acid	To comply with legal requirements
Gravimetric analysis	Residual alcohol content	To comply with legal requirements
	Total dry extract content; ash content; non-volatile reducing substances content; sulphate content	Detection of frauds
Iodometric analysis	Total sulphur dioxide content	To comply with legal requirements
Potentiometric analysis	Chloride content	Detection of frauds
Beta radioactivity ^{14}C	Synthetic acetic acid	Raw material and year of production
Sensory analysis	Odour and flavour attributes	Characterisation; ageing evaluation; quality certification (PGI, PDO); raw materials and production process
HPLC	Phenolic acids	Production process; Origin and technology involved
	Phenolic compounds	Ageing; production in different wood types
GC	Polyalcohol content	Origin
	Acetoin content, methanol, superior alcohols and ethyl acetate	Determination of quality and origin
GC-MS	Volatile aldehydes	Raw material and ageing
	Volatile compounds	Raw material and production process; quality certification (PGI, PDO); ageing
GC-O	Odour impact	Characterisation
NIR	Spectral profile	Raw material and production process; detection of frauds; origin; authentication (PGI, PDO)
MIR	Spectral profile	Ageing; raw material and production process; quality certification (PGI, PDO vinegars)
Fluorescence	Spectral profile	Ageing and authentication (PGI, PDO)
^{1}H-NMR	Spectral profile and vinegar metabolites	Authentication (PGI, PDO) and detection of frauds
	Organic components	Raw material and production process
^{13}C NMR, HMBC, and HSQC	Spectral profile and vinegar metabolites	Authentication (PDO, PGI…)
ICP-OES/ICP-MS	Mineral composition	Geographical origin
IRMS, SNIF-NMR	Site-specific D/H isotope ratio of acetic acid, $^{13}C/^{12}C$ ratio of Acetic acid and $^{18}O/^{16}O$ ratio of water	Detection of frauds: addition of synthetic acetic acid, water or sugar, from plants C_3 or C_4
IRMS	$^{13}C/^{12}C$ isotope ratio of acetic acid	Botanical origin, addition of sugar from C_4 sources
	$^{18}O/^{16}O$ isotopic ratio of water	Addition of water to dried grapes
SNIF-NMR	Site-specific D/H ratio of acetic acid	Botanical origin, addition of synthetic acetic acid
FES, FAAS, AAS	Metallic and trace element components	Production process
Colorimetric techniques	Volatile organic compounds	Production process
E-tongue, E-nose	Aroma and taste signals	Raw materials and ageing

5. Conclusion

The issues mentioned in the sections above are those that have already been identified and remain the most economically viable forms of adulteration at the present time. However, in the future, there could be more problems that should be kept in mind. These problems will most likely concern the growing range of new vinegar types, less common nowadays in the market or the emergence of other food ingredients that can create new, potential areas of deception when used improperly.

The diversity of vinegars in the market and the increase in demand makes it necessary to characterise them to establish quality control parameters. The characterisation of the vinegar covers different objectives including the authentication and classification of the product based on quality criteria. Consequently, there is an increasing need for investigating reliable analytical methods able to detect the possible adulterations and frauds as well as to assess the authenticity of the vinegar.

In recent years, there has been a growing need to develop fast, cheap, robust and effective analytical methods that do not require much sample manipulation such as sensors and spectroscopic techniques (e.g. MIR, NIR, Fluorescence, NMR and UV) coupled to chemometric tools. These techniques take into account both the individual contribution and the interactions of the different components presented in the vinegar, generating a global fingerprint of a food product. However, one of the main disadvantages is their ability to recognise just a limited number of molecules.

Finally, given the complexity of vinegars, and the fact that they are perceived by the consumer in a global way, they must be evaluated from a multivariate point of view. For this reason, a new trend in food authentication based on a combination of more than one of the aforementioned techniques has appeared. This promising methodology known as “data fusion” should be further studied for vinegar authentication.

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ANEXO II

Chapter 27. Fraud, quality and methods for characterization and authentication of vinegars

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27.1 INTRODUCTION

Nowadays there is a growing demand for high-quality food products, one such product being vinegar. In the past, vinegar was considered as a secondary product within the family of fermented products and lacked a recognized quality standard. However, in the last few years a radical change has taken place. Quality vinegar, which until recently was only really appreciated in *haute cuisine* and gastronomy, has seen its demand in households increase worldwide, many consumers now regarding it as a high-quality product. This fact has led to a huge diversity of vinegars appearing on the market with widely differing final sale prices according to their characteristics and quality. This, in turn, has led to research into reliable analytical methods in order to establish criteria for determining a vinegar's quality and origin.

Vinegars are very complex, multi-component mixtures of chemicals and their characterization, by means of their quality and organoleptic properties, requires the determination of a chemical complexity conditioned by both the raw material and the particular elaboration process used and, occasionally, the system of ageing and type of wood used in the process.

Moreover, some vinegars are protected by a legal framework known as Protected Geographical Indications (IGP) or Protected Designation of Origin (PDO). Such certification requires producers to respect the traditional methods of production and to ensure the vinegar's origin. PDO vinegars are high-quality products, produced in a certain area and by specific production procedures, providing them with unique quality characteristics. In Europe there are five geographical indications for vinegars:three from Spain (wine vinegars from the PDOs of *Vinagre de Jerez*, inscribed in 2010; *Vinagre del Condado de Huelva*, inscribed in 2011; *Vinagre de Montilla-Moriles*, inscribed in 2015); and two from Italy:Traditional Balsamic Vinegar of Modena and vinegar from Reggio-Emilia (certified in 2000). These PDO wine vinegars are produced from high-quality wines by a time-consuming traditional process with high production costs. Therefore, not only does the final price increase, the quality does as well. In China the government has awarded PGI certification to Zhenjiang vinegar, Shangxi extra-aged vinegar, Kazuo aged vinegar, Yongchun aged vinegar and Duliu vinegar. The main differences between Chinese vinegars and European vinegars are their raw materials:rice, sticky rice, sorghum, and wheat bran in the case of Chinese vinegars, and wine, cider, fruit juices, malted barley, honey, and pure alcohol in that of European vinegars (Xiong et al., 2016). A PDO or an IGP implies that once the name is registered, it is protected against the marketing of any other competing imitation product seeking to use the reputation of the name of origin (Cocchi et al., 2006). This does, however, lead simultaneously to it being a greater target for fraud and imitations.

Furthermore, the need to determine objectively the appropriate parameters that enable us to characterize and differentiate one vinegar from another, thus ensuring the vinegar's authenticity, is as important as obtaining vinegar of specific quality. The authentication and classification of vinegars, on the basis of assuring their quality and origin, is important for protecting the consumer against being sold an inferior product with a false description; for

ensuring safety in the vinegar industry and, in addition, for defending honest traders from unfair competition by verifying that the vinegar complies with its label description. In recent years, scientific interest in the issue of authenticating high-quality food products has been growing continuously. This interest has been determined mainly by the continuous challenge in the food industry to produce high-quality vinegars and by the need to ensure authenticity and traceability by more objective analytical methodologies with respect to paper certifications. Due to the wide range of parameters to be studied, as well as to the sophistication of the frauds, this is indeed a difficult challenge.

25.2 FRAUDS

Over the years, many vinegar frauds have been perpetrated. One of the first frauds, and one that has been occurring for more than eighty years, is the addition of chemical acetic acid to vinegar. Other long-standing frauds have also occurred in different countries, such as is the addition of cider vinegar to wine vinegar in Switzerland in order to lower production costs (Bourgeois et al., 2006). Due to variations in the legal definition of vinegars from one country to another, other frauds in the vinegar industry have now appeared. For example, while in the European Union, the term vinegar describes 'a product of a double fermentation (alcoholic and acetic fermentation) from substances of agricultural origin', in the USA a 'synthetically-produced acetic acid diluted with water' can also be labeled as vinegar. Another example occurs with vinegars made from wine. In this case, legal definitions in Germany permit the production of vinegar by acetic fermentation from natural ethanol, by diluting acetic acid with water or by blending fermentation vinegar with synthetic acetic acid, or with vinegar made from synthetic acetic acid (Werner and Roßmann, 2015). However, European regulations indicate that wine vinegar can only be produced through the acetic fermentation of wine produced from fresh grapes. Authentic wine vinegar cannot, therefore, contain acetic acids obtained from either petroleum derivatives, wood pyrolysis (synthetic acetic acid) or from the fermentation of sugars not derived from grapes (e.g., derived from beet or cane). Coincidentally, commercializing vinegars produced with alcohol from different origins, as genuine wine vinegar, is one of the most common fraudulent activities in the vinegar industry. This fraudulent practice aims to reduce manufacturing costs and constitutes a fraud to consumers. This adulteration is difficult to detect due to the fact that sometimes the alcohol's provenance is not well-known (Sáiz-Abajo, González-Sáiz, and Pizarro 2005).

Another unfair practice related to wine vinegars is to produce wine and wine vinegar from dried grapes diluted with water. This so-called 'raisin vinegar', commonly produced in some Mediterranean countries by fermenting dried grapes and rehydrating with tap water, cannot be regarded, or labeled, as wine vinegar. Nonetheless, the production of 'wine vinegar' by the above method has been encountered in some Mediterranean countries such as Greece, while it has also been improperly imported into Italy as wine vinegar (Camin et al., 2013). On the other hand, many fraudulent activities also appear with regard to vinegars bearing the label of a protected designation. Whereas the existence of protected origin designations or quality labels in vinegars, very common in the south of Europe, provides a greater guarantee to the product it does, at the same time, encourage the picaresque nature of unfair producers. Although these PDOs strictly regulate their production procedure, the production area, the ageing through traditional practices, and the organoleptic and analytical characteristics – all regularly controlled by council regulations – some adulteration or frauds have occurred. These illegal acts mislead

the consumer and create unfair competition. All too often, however, they are condoned by leading manufacturers, mainly due to the powerful argument of extra profit. A well-known case is that of Traditional Balsamic Vinegar of Modena PDO and the Balsamic Vinegar of Modena PGI. The first is produced by a traditional, time-consuming and expensive production method obeying very strict rules of raw material provenance and production methods, ensuring a high quality. The second is produced industrially and is a much cheaper product made from cooked must, concentrated must and wine vinegar via a complicated process. It is, however, a much quicker process than that employed for Traditional Balsamic Vinegar (Consonni et al., 2008a, 2008b). Due to their different prices, frauds and unfair practices or mislabeling are not infrequent. Thus, many brands of these popular vinegars commercialized in the market are in fact merely a sweetened red wine vinegar with food coloring and not produced using the grapes specified by the denomination. Moreover, they are either not aged at all, or are aged for a short period of time in stainless steel barrels (Werner and Roßmann, 2015). Furthermore, the renowned Spanish wine vinegar PDOs have also suffered – and still do suffer – similar unfair practices such as the falsification of the ageing process or of ageing time length.

The main problem with these high-quality vinegars is that presently certification is obtained by sensory analysis and by a single physiochemical properties determination, such as total acidity, density and dry residue. Certification is undertaken by private corporations using nonobjective analytical techniques in order to determine origin and ageing (Consonni et al., 2008b).

These analytical tools are, therefore, still insufficient to detect common frauds in vinegar and due to the great variety of frauds and their increasing level of sophistication, they have limited abilities of detection. For all of these reasons, there is a need to characterize and establish quality parameters for vinegars with the final aim of establishing a guarantee of authenticity and of combating the current upsurge in frauds that may ultimately have irreversible economic consequences for the honest sector of the industry that complies with the legislation. For this purpose, several groups are working on the characterization and discrimination of different kinds of vinegars, especially in those with high quality and high prices, looking for the most reliable, accurate, robust and economical analytical techniques.

27.3 QUALITY PARAMETERS

As vinegar is not always made from wine, and sometimes apple cider, beer, and grape must are used, the parameters to be evaluated are for purposes of quality and classification change. First, considering that there are different laws or statutes on vinegar for different countries, there are many analytical parameters that could define a vinegar. In spite of this, the common and traditional analytical parameters used to define a vinegar are its acidity and residual ethanol and the acetic acid/ethanol ratio (Solieri and Giudici, 2009). However, it should be taken into account that the total acidity content varies from one vinegar to another, as well as from one country to another. Thus, the United States Food and Drug Administration (FDA) requires that any product called ‘vinegar’ contains at least 4% acidity. The Codex standard proposed a minimum of 6% for wine vinegar and 5% for others due to the fact that the percent of acetic acid present in the product varies according to the raw material used (Ji-Yong et al., 2013; Moros et al., 2008). Acetic acid and ethanol contents change, according to the raw materials used, the fermentation microorganisms, and the technology employed. Mainly,

however, they vary according to the type of vinegar and for this reason, acidity level is not really a measurement of quality.

In general terms, the aspects responsible for food quality are nutritional value, food safety and sensory properties. However, as vinegars are mainly used as a condiment, in their case, quality is strongly determined by sensory properties and the sensory quality of vinegar is mainly determined by its aroma. In addition to acetic acid and ethanol, vinegar contains other constituents which play an important role with regard to its smell, taste and preservative qualities. Those constituents that influence the flavor of vinegars and, therefore, their aromatic composition, originate in, and are influenced by, the raw material, the production process, the constituents formed during fermentation, and, on occasion, those that appear during ageing in wooden barrels.

The raw material provides a large number of relevant compounds for quality, such as characteristic aromatic compounds and polyphenols. This last group of compounds is found in greater quantity in wine vinegars than in other vinegars such as those obtained from apples or honey. These compounds exert a strong influence on the organoleptic properties (color, flavor and astringency) as well as on beneficial properties (Cerezo et al., 2010).

The production process also has a great influence on aromatic composition. Indeed, the species diversity of bacteria involved in acetification has been demonstrated to influence the final different composition of the vinegar (Valero et al., 2005; Tesfaye et al., 2002b). Moreover, the acetification method used in vinegar production also plays an important role in the final aromatic composition. In general, these methods can be divided in two groups:a rapid or submerged process in steel tanks with a submerged culture of bacteria where oxygenation is favored by agitation, or a surface method, also known as the slow method, in which the culture of acetic acid bacteria grows on the surface of the liquid. Most commercialized vinegars are produced by the quick method, whereas the traditional vinegars, such as those with a PDO, are produced by slow acetification processes which usually give rise to a higher quality (Natera et al., 2003; Morales et al., 2001).

Ageing in wood also contributes to the increase in the aromatic complexity of these wine vinegars and it also influences the color of the vinegar, both of which are other important features used by consumers to assess the quality of a food product. During ageing in wooden barrels, chemical modifications occur. These include esterification, condensation and the concentration of compounds due to water evaporation through wood pores. Some compounds are also extracted from the wood, imbuing the final product with specific and singular properties (Marrufo-Curtido et al., 2012). The time and type of ageing (in different kinds of woods) are other sources of variability and greatly affect vinegar quality (Ríos-Reina et al., 2017b; Callejón et al., 2010).

Viscosity is another important parameter in the sensorial quality of some vinegars such as the case of the Traditional Balsamic Vinegar of Modena. Nevertheless, no procedure has yet been established to determine this objectively.

Finally, it is also important that regardless of the vinegar type, adding extracts, sugars, colorings, artificial colorings or preservatives to a vinegar should be also taken into account with regard to quality, due to the fact that their presence could generally be indicative of a lowerquality vinegar.

27.4 CHARACTERIZATION AND AUTHENTICATION OF VINEGARS

Nowadays, the increasing diversity of vinegars on the market and the growing consumer demand for some vinegars and quality condiments have created a need for them to be characterized by establishing specific parameters and providing adequate quality control in order to defend their identity (Cerezo et al., 2008; Liu et al., 2008; Marrufo-Curtido et al., 2012). Moreover, due to the above, these products are becoming greater targets for fraud and they require new tools to combat falsification or mislabeling. Therefore, vinegar characterization aims to protect consumers against the commercialization of products of a quality inferior to that declared in its description, as well as defending honest producers against unfair competition. Vinegar, therefore, like all other food products, must comply with quality specifications and must bear a label that describes the product faithfully.

In order to set up validated methods able to ensure the authenticity of food and differentiate defective or adulterated vinegars from the genuine article, many parameters have been studied in several pieces of research and by applying several different techniques. In this context, the polyalcohol content was demonstrated to be useful in ascertaining a vinegar's origin, in the case of suspected wine vinegar adulteration with less expensive alcohol vinegar (Antonelli et al., 1997). Moreover, some volatile compounds such as ethyl propionate and acetoin have also been used to distinguish between quality and defective or adulterated samples of wine vinegar (Chinnici et al., 2009; Durán-Guerrero et al., 2015). Another group of widely-studied compounds are phenols present in wine vinegars due to their natural occurrence in grapes, or due the vinegar's contact with wood during the ageing process. These compounds have been studied as possible indicators of the geographical origin of the substrate, the elaboration method involved and their ageing (García-Parrilla et al., 1997). Moreover, the ratio of D/L proline has been studied in order to evaluate ageing time.

Traditional ageing, entailing a greater investment in time and higher production costs, is important due to the high quality of the vinegar produced. Good results have been achieved with regard to differentiating between quick acetification and the traditional methods using analytical parameters such as acidity, total extract, glycerol, alcohol, sulfates and minerals. If interest is to be focused on the characterization of a particular kind of vinegar, such as differentiating between Balsamic Vinegar of Modena and Traditional Balsamic Vinegar of Modena, the analysis of D- and L- amino acids as (R)- and (S)-acetoin levels has been demonstrated to be effective (Chiavaro et al., 1998).

27.5 METHODS FOR CLASSIFYING AND AUTHENTICATING VINEGARS

Due to the wide diversity of types of vinegar produced from different raw materials and by different production processes there are therefore, many vinegars with different final qualities on the market. This means that there is a growing need to investigate reliable analytical methods which are able to determine quality and origin. As well as assessing a vinegar's authenticity, these methods have to be able to detect possible adulterations and frauds.

In general, these methodologies can be grouped into two types:sensory analysis and physicochemical analysis. Due to the fact that a vinegar's quality is mainly associated with its aroma, sensory assessment is the first method to take into account. Sensory analysis is a powerful tool used to appreciate vinegar quality from the point of view of the producer, researcher, or the consumer. However, although sensory analysis plays a major role in the

acceptability of vinegar from the point of view of the consumer, instrumental analysis is also needed to ensure final quality and to fulfill legal requirements. Thus, the other type of techniques to study in the field of characterization and authentication are those that analyze the vinegars' physicochemical characteristics. In turn, these physicochemical techniques can be grouped according to two strategies:the first consists of those techniques able to analyze one or more specific components that could be markers of a specific vinegar (targeted methods), while the other strategy is formed by those techniques that try to obtain the "fingerprint", or profile, of a vinegar analyzed by one technique and then building category models by using chemometric tools (untargeted or nontargeted methods) (Cocchi et al., 2004).The procedure steps of a targeting and untargeting vinegar analysis is schematized in Figure 27.1.

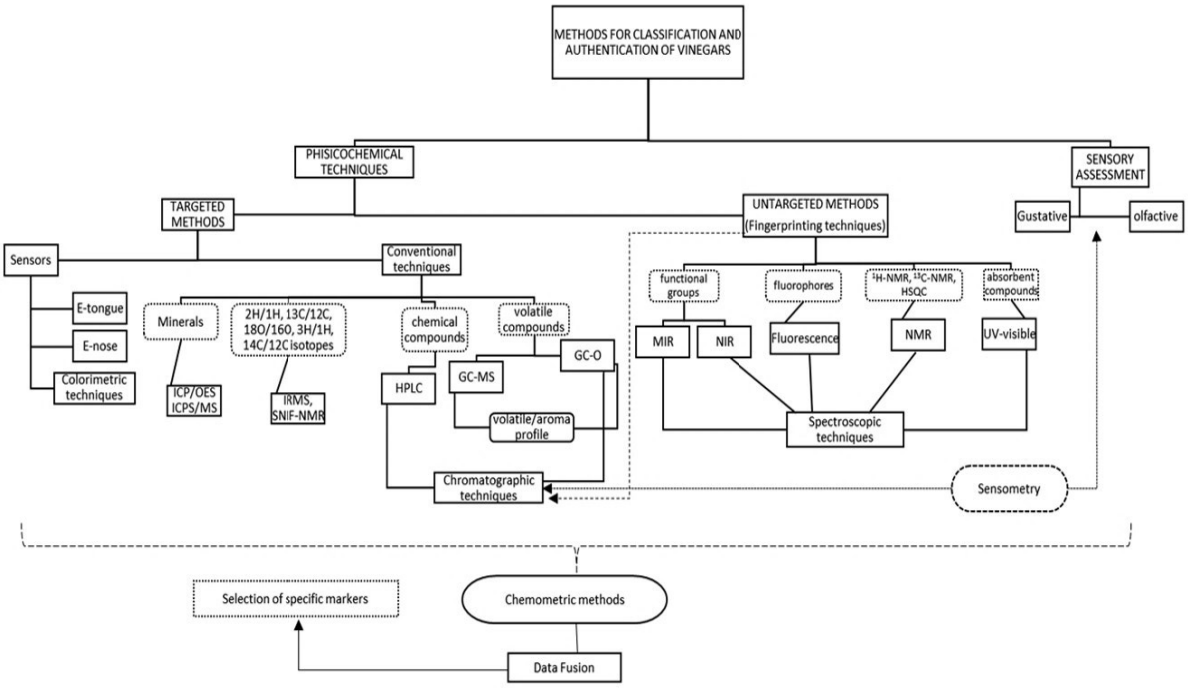

FIGURE 27.1. The procedure/strategy of targeting and untargeting techniques in vinegar analysis

With regard to the first strategy, widely-used conventional methods for characterizing and authenticating vinegars include the analysis of ash content, phosphorous content and acidity, as well as determining certain amino acids, by-products of the acetobacter fermentation, substances derived from raw materials, trace elements and metals content and, in some vinegars, the phenolic compounds derived from ageing in wood. The determination and quantification of these compounds have been performed by such methodologies as gas chromatography-mass spectrometry (GC-MS) (Plessi et al., 2006), high-performance liquid chromatography-mass spectrometry (HPLC-MS) (Tesfaye et al., 2002a; Cerezo et al., 2008, 2010), or enzymatic methods (Verzelloni et al., 2007).

The main strength of the second, more recent, strategy consists of taking into account both the individual contribution and the interactions of the different components presented in vinegar modeling, in other words, the total complexity of the food matrix (Cocchi et al., 2004). In this case, the methodologies being studied are several spectroscopic techniques such as mid- and near-infrared spectroscopies (MIR, NIR) (De la Haba et al., 2014; Zhao et al., 2011;

DuránGuerrero et al., 2010; Ríos-Reina et al., 2017b, 2018b); fluorescence spectroscopy (Ríos-Reina et al., 2017a; Callejón et al., 2012), nuclear magnetic resonance (NMR) (Fotakis et al., 2013; Papotti et al., 2015) and even GC-MS (Ríos-Reina et al., 2018a; Casale et al., 2006), when looking at the total volatile profile of a sample.

27.5.1 Sensory analysis

Sensory analysis is a valuable tool. In other words, a foods' organoleptic properties are analyzed by our senses. Provided that it is carried out with trained assessors using methodological criteria that enable results to be processed statistically (Gerbi et al., 1997), sensory analysis has proven to be a simple and reliable tool for assessing the quality of vinegars. However, sensory analysis in the case of vinegar is particularly arduous because of the aggressive taste and smell of the product due to acetic acid contributing overwhelmingly to the overall sensation. Therefore, the appropriate sensory methodology must be clearly defined and the attributes used in discriminant or descriptive analysis must be precise and well-recognized by the panel (Tesfaye et al., 2009).

Sensory vinegar analysis can be performed by olfactive and gustative analyses. In gustative analysis, there are also different methodologies such as preparing the vinegar in a way that most resembles how it is normally consumed or testing vinegar as is, using wine glasses. This latter is the usual procedure in vinegar cellars in order to perform sensory analysis (Tesfaye et al., 2002). Moreover, with regard to the olfactory procedure, there are also different tests such as the triangle test, paired comparison test, preference test, etc.

The sensory characterization of vinegars has been widely performed for many years. Thus, Gerbi et al., (1997) performed a sensory analysis of vinegars from different sources showing that sensory analysis enables the different sources of vinegars, such as alcohol and apple vinegars to be discriminated from wine vinegars on the basis of only seven sensory parameters. Some years later, Tesfaye et al., (2002) developed a sensory evaluation of Sherry wine vinegars according to changes that occurred during ageing. This study showed clearly that both aroma intensity and quality increased with ageing. Morales et al., (2006) also studied the importance of the sensory profile of wine vinegars produced by accelerated ageing compared with those elaborated by a traditional method (Sherry vinegar), and therefore, the ability to differentiate sensorily a "rapid" vinegar from a high-quality wine vinegar.

Much previous research into the monitoring of vinegar quality has been based on a range of sensory analyses. Moreover, in some vinegars quality control is mainly based on their sensory properties, as is the case for Traditional Balsamic Vinegar of Modena. Therefore, its quality certification is obtained on the basis of sensory evaluation, together with a few chemical and physical analyses, such as total acidity, density and dry extract (Lalou et al., 2015; Masino et al., 2008; Hillmann et al., 2012).

27.5.2 Physicochemical analyses

In spite of the fact that the quality of vinegars has been evaluated by using a trained sensory panel, a more rapid and objective characterization is being studied and performed by instrumental measurements. Physicochemical analyses are commonly used for complying with legislative requirements regarding a vinegar's quality, safety and characterization. In this context, several studies which aim to characterize or differentiate vinegars are to be found in the literature (Marrufo-Curtido et al., 2012; Xiao et al., 2011; Cocchi et al., 2007; Ortiz-Romero

et al., 2018). These studies can be structured by means of the analytical method applied, enabling an evolution over the years in terms of the methodologies studied with the aim of characterizing vinegar and controlling its quality, to be observed. They can, moreover, be grouped according to the above mentioned strategies in targeted and non-targeted or untargeted methods. This classification of techniques can be seen in Figure 27.2.

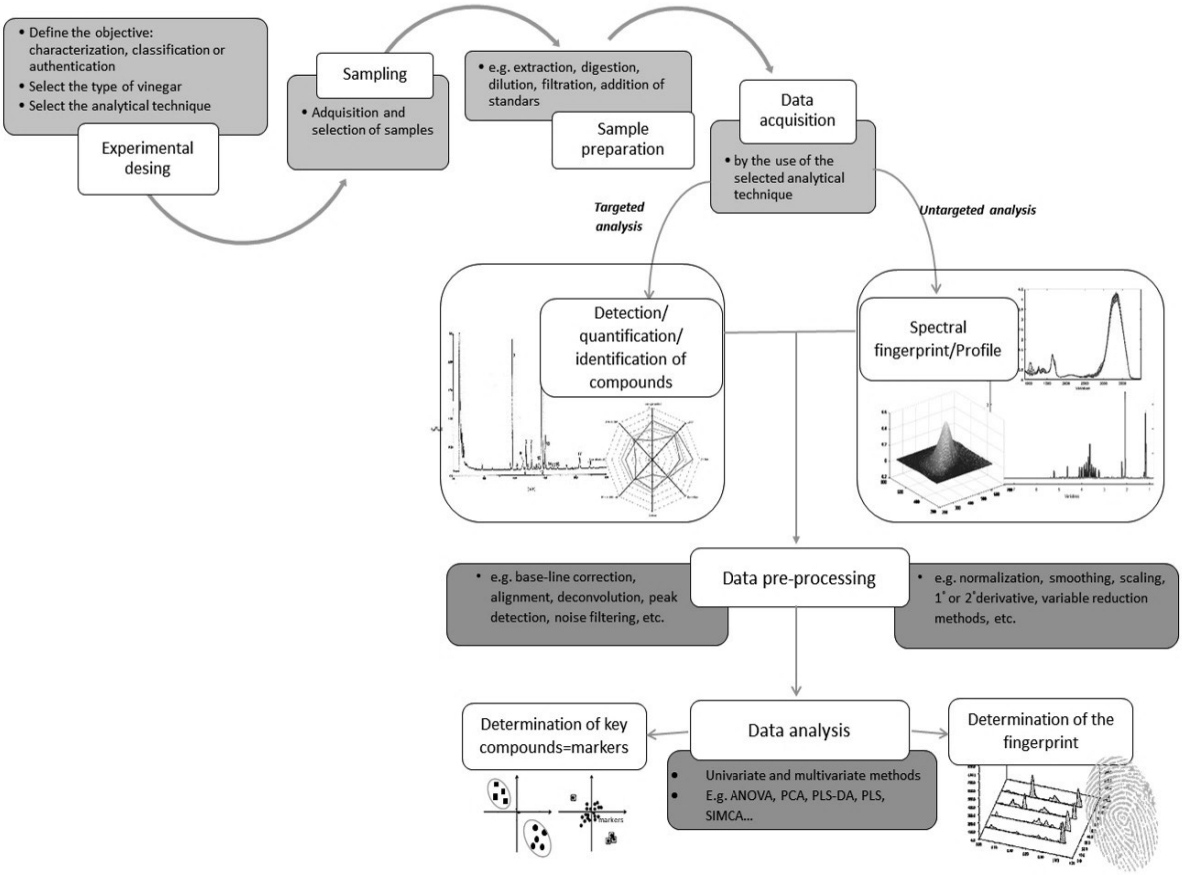

FIGURE 27.2. Schematic classification of techniques used for characterizing and classifying vinegars.

27.5.3 Chromatographic techniques

Traditionally, chromatographic techniques have been applied for determining certain vinegar compounds. It can be useful for characterizing, classifying or detecting adulterations in vinegars.

On the one hand, HPLC has been widely applied for analyzing compounds, such as phenols, that seem to be an important group of substances for differentiating vinegars from different origins and produced by different acetification methods (Garcia-Parrilla et al., 1994, 1997). These compounds have also been linked to the ageing stage and the type of wood used in order to differentiate the vinegars' different qualities (García-Parrilla et al.,1999; Tesfaye et al., 2002a; Cerezo et al., 2008, 2010).

On the other hand, GC-MS has been the most widely employed technique for analyzing a vinegar's volatile composition which is directly related to vinegar quality, as well as for determining certain relevant compounds. It was, therefore, used to determine poly-alcohols for characterizing the vinegars from different botanical origins or for detecting a suspected adulteration of wine vinegars with less expensive alcohol vinegars (Antonelli et al., 1997).

Moreover, GC-MS, coupled with different prior extraction steps, has been applied for assessing volatile aldehydes as discriminating parameters in quality vinegars (Durán-Guerrero et al., 2015); for characterizing and classifying different vinegar types (white and red, balsamic, sherry, strawberry and cider vinegars) on the basis of their volatile composition (Pizarro et al.,2008; Ubeda et al., 2016; Cocchi et al., 2004; Cirlini et al., 2011; Chinnici et al., 2009) and for differentiating quality vinegars with a PDO or PGI (Cocchi et al., 2004; Marrufo-Curtido et al., 2012; Chinnici et al.,2009; Ríos-Reina et al., 2018a).With regard to this last issue, Chinnici et al., (2009) demonstrated that by applying GC-MS analysis, short-chain fatty acids, furanic compounds, enolic derivatives, and some esters were responsible for discriminating three different PGI (Traditional Balsamic Vinegar of Modena, Balsamic Vinegar of Modena, and Sherry Vinegar). In a similar way, Marrufo-Curtido et al., (2012) also used the GC-MS methodology for characterizing the volatile composition of the same three different PGI. Moreover, Cirlini et al., (2011) used GC-MS to distinguish the less matured Balsamic Vinegar of Modena from the aged ones. Ríos-Reina et al., (2018a) studied different sampling methods coupled with GC-MS to assess and compare their applicability in analyzing the volatile composition of Spanish PDO wine vinegars with the final aim of discriminating them. With regard to Chinese vinegars, GC-MS has also been applied to differentiate them according to type, fermentation method, and production area (Xiao et al., 2011; Yu et al., 2012; Xiong et al., 2016).

However, all of the volatile compounds present in a vinegar do not make the same contribution to a vinegar's overall aroma. In this context, gas chromatography coupled with olfactometry (GC-O) is the technique used to determine those compounds, known as impact odorants, which have a real impact on the aroma of a vinegar. In spite of having been demonstrated to be a valuable method for the selection of odor components from complex mixtures and for identifying the active odor compounds, little research can be found in the literature regarding the application of this technique in vinegars. Thus, only Sherry and some Chinese vinegars have been analyzed by this technique (Zhou et al., 2017; Callejón et al., 2008a, 2008b).

Finally, in spite of the fact that chromatographic techniques are time-consuming and expensive, it should be taken into account that in recent years, the development of chemometric tools (i.e. Multivariate Curve Resolution, Parallel Factor Analysis, etc.) are opening up a new way of solving chromatographic problems and of improving the interpretation of complex data by means of a quick and accurate analysis, as well as opening up a new way of performing an untargeted analysis (Hantao et al., 2012; Casale et al., 2006; Ríos-Reina et al., 2018a; Cocchi et al., 2007).

27.5.4 Spectroscopic techniques

Rapid scientific and technological advances in food authenticity determination have taken place in recent years due to the fact that, in many cases, an unequivocal decision on the authenticity of a sample with conventional methods is not possible. Moreover, most of the conventional analytical methods developed for vinegar characterization and quality control are expensive, destructive, and time-consuming, as well as requiring skilled operators and having a high environmental impact. For this reason, rapid, inexpensive, non-destructive and direct methodologies based on non-targeted techniques are becoming more interesting as an approach to authentication (Ríos-Reina et al., 2017a, 2017b, 2018b). Therefore, those methodologies able to provide "fitness for purpose" results, taking into account aspects such as

the importance of time against accuracy achieved, are currently becoming a developmental trend in analytical chemistry. In contrast to quantitative results, these methods are mainly based upon qualitative aspects. Within this group of techniques there is great interest in the application of spectroscopic techniques based on infrared (IR), fluorescence or NMR spectroscopy in order to enable more objective, rapid, and less expensive vinegar quality assessments (Versari et al., 2011). These techniques are the most commonly-used for vinegar fingerprinting due to the fact that they fulfill the abovementioned characteristics while also allowing several properties to be determined simultaneously by taking into account both the individual contribution and the interactions of the different chemical components in vinegars (Cocchi et al., 2004). Additionally, other reasons for the interest in these methodologies are that, except for calibration, they do not require specially trained workers.

In this sense, vibrational spectroscopic techniques, such as Near-Infrared spectroscopy (NIR) and Fourier Transform mid-infrared spectroscopy (FTIR) have been demonstrated to meet the above characteristics. NIR spectroscopy has been used for performing a simultaneous in-line monitoring of ethanol and other compounds relevant to vinegar quality, as well as monitoring the production process, allowing particular corrective actions to be assessed in the shortest possible time. Several research works have also demonstrated its usefulness in the classification of vinegar samples according to the raw material of origin and elaboration process. Thus, Saiz-Abajo et al., (2004) used NIR spectroscopy to classify wine vinegar and alcohol vinegar in northern Spain with calibration and prediction classification rates of 85.7% and 100%, respectively, as well as demonstrating the suitability of this technique for classifying vinegars from eight different raw materials and with respect to different processing methods such as must addition, fermenting or ageing in wood (Sáiz-Abajo et al., 2004). It has, moreover, been successfully applied in the determination of total acids, non-volatile and volatile acids, organic acids, L-proline, solids, ash and chloride in vinegar, which is useful for monitoring vinegar processes on an industrial scale (Sáiz-Abajo et al., 2006). NIR spectroscopy has also been used as a rapid classification method for the geographical origin of mature vinegars (Lu et al., 2011); for discriminating fermented vinegar from blended vinegar (Fan et al., 2011) and for detecting adulterated vinegars (SáizAbajo et al.,2005). Furthermore, it has been investigated as a method for authenticating and classifying PDO wine vinegars (De la Haba et al., 2014; Ríos-Reina et al., 2018b).

Mid-infrared measurement (MIR), usually using Fourier Transform based instruments (FTIR), has also been developed for the the individual estimation of compounds of interest in vinegars. When compared with NIR spectroscopy, FTIR spectroscopy is an analytical technique that provides a greater amount of chemical information in terms of the chemical assignment of observations. Although NIR spectroscopy is faster, easier to implement and easy to use, FTIR has provided good results in the analysis of Spanish PDO wine vinegars in terms of monitoring their ageing and sweetness categories (Ríos-Reina et al., 2017b). MIR spectra have also been used to discriminate between traditional balsamic vinegar and other vinegars (Del Signore 2000), as well as being used as a tool for predicting a vinegar's sensory quality with a good correlation ($r = 0.88$), making it a possible substitute for trained panelists (Versari et al., 2011). Moreover, midFT-IR spectra have been studied in the same way as NIR for testing its ability to classify vinegars from different raw materials and with or without ageing in wood (Durán-Guerrero et al., 2010).

Simultaneously, fluorescence spectroscopy has been also investigated as an alternative quality control tool for vinegars. Even though fluorescence is one of the oldest analytical methods used (Valeur 2001), it has recently become quite popular as a tool in food technology. Thus, Callejón et al., (2012) and Ríos-Reina et al., (2017a) studied fluorescence excitation–emission spectroscopy combined with adequate multi-way methods and demonstrated this method's ability to characterize and classify the three Spanish PDO wine vinegars according to their protected designation of origin, as well as their categories (aged and sweet). In the same way as the abovementioned techniques, and due to the successful results obtained for quality control in other food products (Acevedo et al., 2007; Azcarate et al., 2013), ultravioletspectroscopy was another approach studied for use in vinegar discrimination and classification (Xie et al., 2011).

NMR spectroscopy also has offered many advantages such as the simultaneous and rapid determination of different vinegar metabolites. This makes the technique another useful fingerprint method for food authenticity and quality control. Moreover, it also offers a remarkable selectivity and identification of unknown compounds with high reproducibility and repeatability. NMR spectroscopy has, furthermore, the ability to furnish structural and quantitative information on a wide range of chemical species in a single experiment (Fotakis et al., 2013). In this context, proton nuclear magnetic resonance (^{1}H-NMR) has been used for the rapid determination of compounds such as carbohydrates, organic acids, alcohols, polyols and volatile substances relevant to vinegar discrimination (Caligiani et al., 2007). Moreover, Papotti et al., (2015) used ^{1}H-NMR, Carbon-13 nuclear magnetic resonance (C-NMR), and H-C heteronuclear single quantum coherence (HSQC) spectra, coupled with multi-variate statistical data analysis in the characterization of Balsamic Vinegar of Modena and Traditional Balsamic Vinegar of Modena. This study showed that the signals of 5-HMF, α and β-glucopyranose, malic, succinic, tartaric and acetic acids, 6-acetyl glucose, and the glucose and fructose region were the most statistically significant variables for discriminating the balsamic vinegars and for monitoring the ageing process (Papotti et al., 2015). Consonni et al.,(2008b) also studied the power of ^{1}H-NMR in combination with chemometrics in characterizing and discriminating Balsamic and Traditional Balsamic Vinegar and Vinegar of Modena, as well as the applicability of ^{13}C NMR for determinating the fraudulent practices present in unknown Traditional Balsamic Vinegar of Modena samples (Consonni et al.,2008a). Boffo et al., (2009) demonstrated the potential of the ^{1}H-NMR spectroscopic approach in discriminating Brazilian vinegars according to their raw materials such as wine, apple and alcohol/grain vinegars by finding those components which have the greatest influence in enabling them to be separated from each other. Finally, a novel NMR approach for the classification of Balsamic Vinegars of Modena has recently been studied (Graziosi et al., 2017). It consists of applying a two-dimensional NMR method in order to obtain an indirect indicator of authenticity and a quality control tool. The one-dimensional technique has been widely applied in this field due to having a simpler acquisition procedure and very competitive time consumption. However, although the two-dimensional NMR method usually requires a longer acquisition time compared to one-dimensional, Graziosi et al., (2017) demonstrated that the real advantage of the two-dimensional technique was that it enabled a higher resolution to be gained in the presence of overlapping signals and crowded resonances in the case of very complex matrices such as vinegars.

In spite of the advantages of these techniques, it should be taken into account that spectral data consist of thousands of variables which can be difficult to interpret without the

help of chemometrics (Lohumi et al., 2015). In fact, multivariate analytical methods are able to reduce the dimensionality of the data to a smaller number of components, concentrating the maximum information under study – one of the requirements in spectrum processing. Another advantage of using chemometrics is the ability to obtain a complete profile or fingerprint of a sample analyzed by some of the abovementioned techniques. For these reasons, nowadays most of the studies of food product characterization through spectroscopic techniques use chemometric tools with successful results (Duarte et al., 2004; Karoui and De Baerdemaeker, 2007; Consonni et al., 2008b; Erich et al., 2015; Fotakis et al., 2013; Sinelli et al., 2010; Mazerolles et al., 2002; Maggio et al., 2010; Ballabio and Todeschini, 2009; Ríos-Reina et al., 2018b). Chemometric tools are even used with chromatographic techniques (Ríos-Reina et al., 2018a; Hantao et al., 2012).

27.5.5 Sensors

One alternative technique developed to substitute the perception of human senses is the use of 'artificial sensors'. The objective of sensor technology is to emulate human senses and to predict sensory scores of food by providing signals related to the sensory attributes, together with suitable multivariate pattern recognition techniques (Borràs et al., 2015). The most common sensor devices introduced as effective alternatives to conventional approaches used for the taste and odor analyses of food are electronic noses (E-nose), electronic tongues (E-tongue) and colorimetric techniques whose responses are correlated to aroma, taste and visual attributes, respectively (Borràs et al., 2015).

In terms of vinegar quality assessment, E-nose was applied by Anklam et al., (1998) as a rapid tool for discriminating the industrially-produced *Aceto Balsamico di Modena* from the traditionally-produced *Aceto Balsamico Tradizionale di Modena* – and even to discriminate between them on the basis of the age of the sample. The E-nose method was also attempted in order to characterize the aroma of Chinese vinegar (Zhang et al., 2006, 2008). Unfortunately, acetic acid is harmful to the sensors in an electronic nose, so the device was improved by Guan et al., (2014) by developing a novel electronic nose system based on a colorimetric sensor array made from metalloporphyrin materials and pH indicators printed onto silica gel plates. This new E-nose demonstrated its usefulness in characterizing and identifying the volatile organic compounds (VOCs) of vinegars fermented from different raw materials (Guan et al., 2014). Some other variations have also been carried out to improve the E-nose device, such as applying mass spectrometry (MS) as a sensing element for E-nose (Vera et al., 2011; Jo et al., 2016). A more recent study demonstrated the application of E-tongue, E-nose, and MS-E-nose for discriminating aged vinegars in three types of vinegars (Chinese, Japanese black vinegar and Italian balsamic vinegar), prepared with different raw materials and with different years of ageing (Jo et al., 2016). Finally, Betto et al., (2016) also developed a new sensory device, called the Small Sensor System (S3), coupled with enfleurage. It appears to be a very easy-to-use, fast, accurate, lowpower-consuming, cost-effective and portable tool that could become a valuable alternative to the classic, expensive methods for characterizing aromatic profile and evaluating quality. The results obtained in the characterization of the aromatic profile of Balsamic Vinegars and evaluation of their quality have demonstrated its usefulness (Betto et al., 2016).

In general, the application of these techniques combined with an appropriate pattern recognition system can generate a global fingerprint for a food product. However, one of the main disadvantages is their ability to recognize a limited number of molecules only.

27.5.6 Other techniques

Additional parameters for vinegar authentication have also been studied. Inductively-coupled plasma optical-emission (ICP-OES), atomic absorption spectrometer (AAS), flame absorption (FAAS) and emission spectrometry (FES) are applied to determine the mineral composition and the trace metal contents in vinegars. These techniques have been useful for determining geographical provenance or for classifying vinegars produced from different raw materials or by different acetification processes (Del Signore, Campisi, and Di Giacomo1998; Paneque et al., 2017; Guerrero et al., 1997).

Moreover, analyzing the isotope ratios of the bioelements ($^{2}H/^{1}H$, $^{13}C/^{12}C$, $^{18}O/^{16}O$) has shown to be useful for providing the information for proof of vinegar authentication. In fact, the Compendium of International Methods of Analysis of wine vinegars includes isotopic mass spectrometry (IRMS) for monitoring wine vinegar parameters:one for determining the isotopic ratio $^{13}C/^{12}C$ of acetic acid (OIV-OENO 510-2013) and another for determining the $^{18}O/^{16}O$ isotopic ratio of water in wine vinegar (OIV-OENO 511-2013). Therefore, the $^{13}C/^{12}C$-isotope ratio of the acetic acid can indicate if the source of the acetic acid and the grape sugars is truly grape (wine) ethanol or wine must, or other ethanol made from fermentation of some other cheaper agricultural products (cereal, potato starch, beetroot or sugarcane), the so-called synthetic acetic acid. The isotopic ^{18}O analysis has also shown to be able to detect the fraudulent addition of external water as a method for reducing the acetic degree in the resulting wine vinegar or to differentiate a wine vinegar produced from fresh grapes from a vinegar produced using dried grapes to which water has been added (Camin et al., 2013). Moreover, studies of the C and H stable isotope ratios have shown a strong capability to identify synthetic vinegars and distinguish C3 and C4 derived products, being useful for detecting common vinegar adulterations that occur when cheaper raw fermentation materials than those declared on the label are used (Perini et al., 2014). This methodology has been also used to control the provenance of vinegars. Thus, the study of the C–O isotope fingerprint for different geographical provenances of Spanish wine vinegars has recently been undertaken (Ortiz-Romero et al., 2018). As well as the analysis of stable isotope ratios D/H and $^{13}C/^{12}C$ in ethanol and acetic acid, the isotope analysis of $^{18}O/^{16}O$ in water and the multi-element (C, H, O) stable isotope analysis have been studied in order to evaluate the authenticity of balsamic vinegars (Perini et al., 2014). This type of analysis is also being used as a potential geographical marker for vinegars (Raco et al., 2015).

In addition to isotopic analysis, another isotopic method, called SNIF-NMR (site-specific natural isotopic fractionation-nuclear magnetic resonance spectrometry) has been studied in terms of its ability to determine the origin of vinegars. It has demonstrated its applicability in determining synthetic acid added to vinegar and, more generally, to identify the raw materials or the botanical origin of a vinegar (wine, apple, malt, cane or beet alcohol, etc.). Indeed, it has even been used to determine the origin of the grapes (Solieri and Giudici, 2009).

Finally, given the complexity of vinegars and the fact that they are perceived by the consumer in a global manner, they must be evaluated from a multivariate point of view. Vinegar's quality is derived from a complex combination of characteristics, so analytical measurements for a single compound or technique cannot be completely correlated with quality. For the same reason, frauds can be perpetrated by altering the amount of many components of different natures. Therefore, the use of models that rely on chemometrics and consider the contribution of multiple components or effects can be more promising. For this

reason, a new trend in food authentication, based on a combination of more than one of the abovementioned techniques, has appeared (Borràs et al., 2015). In this context, combined with the rapid, reliable spectroscopic and chromatographic techniques discussed above, multivariate analysis provides more defined information concerning the stated quality of food. This is useful for distinguishing between food samples and it facilitates authenticity determination (Borràs et al., 2015; Silvestri et al., 2013; Natera et al., 2003). Therefore, to this end, a methodology known as “data fusion” has been developed. By means of this method, more accurate knowledge about a sample is provided, entailing less classification error and better predictions than a single technique. Currently many research works on the combination of different kinds of data aiming to provide food authentication are to be found in the literature (Vera et al., 2011; Silvestri et al., 2013, 2014; Borràs et al., 2015; Márquez et al., 2016; Di Anibal et al., 2011). However, in spite of the promising results obtained with other food matrices, there is still a lack of studies with regard to vinegar samples (Natera et al., 2003). Hence, vinegar authentication by means of data fusion strategies requires further study.

[TABLE 27.1 near here]

27.6 CONCLUSIONS

The diversity of vinegars on the market and the increased demand makes it necessary to characterize them in order to establish quality control parameters. Vinegar characterization is a response to different objectives, including quality-based authentication and classification criteria. Consequently, there is an increasing need to investigate reliable analytical methods that are able to detect possible adulterations and frauds, as well as to assess the authenticity of the vinegar. In general, these methodologies can be grouped in two types:sensory analysis and physicochemical analysis.

Since aroma is one of the main quality indicators, sensory analysis is a powerful tool used for appreciating vinegar quality. Indeed, eventhough it requires the screening, selection and training of the testing panel in order to obtain reliable results, it is the first method to consider.

With regard to physicochemical techniques, commonly used methods for the characterizing and classifying vinegars are techniques such as chromatographic and spectrometric techniques (HPLC, GC, ICP-OES, AAS, FAAS and FES, etc.). These include the determination and quantification of single compounds (e.g. volatile compounds, polyphenols, minerals, stable isotopes, etc.). Such techniques are time-consuming, expensive, and laborious and require highlytrained people. However, thanks to the development of chemometrics, this latter is opening up a new means of obtaining more information by performing an un-targeted analysis.

On the other hand, in recent years, there has been a growing need to develop fast, cheap, robust and effective analytical methods that do not require a mere sample manipulation, such as that performed by sensors and spectroscopic techniques (e.g. MIR, NIR, Fluorescence, NMR and UV) coupled to chemometric tools. These techniques take into account both the individual contribution and the interactions of the different components present in the vinegar, generating a global fingerprint for a food product. However, one of the main disadvantages is their ability to recognize a limited number of molecules.

Finally, given the complexity of vinegars and the fact that they are perceived by the consumer in a global manner, they must be evaluated from a multivariate point of view. For this reason, a new trend in food authentication based on a combination of more than one of the

abovementioned techniques has appeared. This promising methodology, known as “data fusion”, should be further studied in order for it to be applied to vinegar authentication.

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TABLE 27.1
Summary of the methods for classification and authentication of vinegars found in the literature

Type/Technique	Analyzed parameter	Characteristics/requirements	Vinegar type	Aim	References
SENSORY ANALYSIS					
Olfactive and/or gustative	Odor and flavor attributes	*Advantages:* - Low costs - Effective for assessing quality *Disadvantages:* - Subjective analysis - Training the panel - Limited vinegar samples examined at each tasting session	Wine vinegars (Sherry vinegars, red wine vinegars, white wine vinegars, etc.)	Characterization and differentiation according to raw materials and production process. Aging evaluation	Tesfaye et al. 2002; Morales et al. 2006; Gerbi et al. 1997
			Balsamic vinegars and Traditional Balsamic Vinegar of Modena	Quality certification Optimization of the taste profile Characterization	Lalou et al. 2015; Masino et al. 2008; Hillmann et al. 2012
CHROMATOGRAPHY					
HPLC	Phenols, amino acids, acids, alcohols, etc.	*Advantages:* - Robust and widely applied - High resolution, sensitivity and specificity - Identification of compounds *Disadvantages:* - Extraction steps - Time and solvent consuming - Standards - Trained analysts - Baseline drifts, co-elution and overlapped peaks - Required data processing	Wine vinegars	Differentiation of origins and different acetification methods Determination of aging time and conditions	García-Parrilla et al. 1994, 1997, 1999; Tesfaye et al. 2002a; Cerezo et al. 2008, 2010;
GC/GC-MS	Volatile compounds		Wine, white and red, balsamic, sherry, cider, PDO, IGP vinegars	Characterization and classification of vinegars according to raw material, PDO and origin. Detecting adulterations Classification according to aging and maturation	Antonelli et al. 1997; Durán-Guerrero et al. 2015; Pizarro et al. 2008; Cocchi et al. 2004, 2007; Marrufo-Curtido et al. 2012; Chinnici et al. 2009; Ríos-Reina et al. 2018a; Cirlini et al. 2011; Casale et al. 2006;
			Chinese vinegars	Differentiation of types, fermentation methods, and production area Discrimination of PGI	Xiao et al. 2011; Yu et al. 2012; Xiong et al. 2016
GC-O	Odor impact		Sherry vinegars	Characterization	Callejón et al., 2008a, 2008b
			Chinese vinegar		Zhou et al. 2017
SPECTROSCOPY					
NIR	Chemical groups and fundamental	*Advantages*:	White, red wine vinegars, aged, sherry, Modena,	Classification according to raw material and elaboration process	Sáiz-Abajo et al., 2004; Sáiz-Abajo et al., 2005

	structural information	- No sample preparation/ Direct analysis - No trained analysts - Faster acquisition of spectra - Low costs - Reliable detection - Allow sample fingerprinting - Non-destructive *Disadvantages:* - Difficult identification of compounds - Require data pre-processing and chemometrics	balsamic, malt, cider and molasses vinegars	Detection of adulterated vinegars	
			Chinese vinegars	Discriminating fermented vinegar from blended vinegar and the geographical origin of mature vinegars	Lu et al. 2011; Fan et al. 2011
			Wine vinegars	Authentication and classification method for vinegars with a PDO	De la Haba et al. 2014; Ríos-Reina et al. 2018b
MIR	Chemical groups and fundamental structural information		Wine vinegars, white and red wine, cooked must and cider vinegars and PDO vinegars	Controlling high quality vinegar categories Classification according the raw material and ageing	Ríos-Reina et al. 2017b; Durán-Guerrero et al. 2010
			Balsamic and traditional vinegars	Classification and prediction of the vinegar sensory quality	Del Signore, 2000; Versari et al. 2011
Fluorescence	Fluorophores (Cumarins, phenols, flavonols, Vitamin B2…)		PDO wine vinegars Aging categories	Characterization and classification	Callejón et al. 2012; Ríos-Reina et al. 2017a
UV	Absorbent species (polyphenolic and acid compounds)		Rice, mille, black rice, sticky rice, wheat bran, barley, sorghum, pea, mulberry vinegars	Discrimination and classification according to raw material and fermentation modes	Xie et al. 2011
NMR	Vinegar metabolites (carbohydrates, organic acids, alcohols, polyols and volatile substances)	*Advantages:* - Quick sample preparation - Nondestructive analysis - Great deal of information and quantitative data in a single experiment - Sample fingerprinting - Unique internal standard required *Disadvantages:* - High instrumentation costs - Trained analysts	Traditional and Balsamic vinegars; wine, apple, rice, malt and tomato vinegars; Brazilian vinegars	Authentication and discrimination according to raw material and quality Detection of frauds and quality control	Caligiani et al. 2007; Boffo et al. 2009; Papotti et al. 2015; Consonni et al. 2008a, 2008b; Graziosi et al. 2017
SENSORS					
E-nose	Signals related to aroma	*Advantages:* - Easy-to-use, - Fast, accurate and low power	Balsamic and Traditional balsamic vinegars	Discriminating and determination of aging	Anklam et al. 1998
			Chinese vinegars	Characterization	Zhang et al. 2006, 2008; Jo

		consumption			et al. 2016
E-tongue	Signals related to taste	- Cost-effective and portable tool - Global fingerprint *Disadvantages:* - Limited number of molecules determined - Difficult identification of compounds	Chinese, Japanese and Italian balsamic vinegar	Discriminating aging and raw materials	Jo et al. 2016
Colorimetric techniques	Signals related to visual attributes		Chinese vinegars	Characterization and discrimination according to raw material	Guan et al. 2014
Small Sensor System (S3)	Lipophilic volatile compounds		Balsamic and Italian vinegars	Aromatic profile characterization and quality evaluation	Betto et al. 2016; Anklam et al. 1998
OTHERS					
ICP-OES/ICP-MS	Mineral composition	*Advantages:* - Rapid multi-element fingerprint - Excellent detection limits *Disadvantages:* - Pretreatment methods - Trained analysts - High cost	PDO wine vinegars	Characterization and discrimination of geographical origin	Paneque et al. 2017
FES, FAAS, AAS	Metallic and trace element components	*Advantages:* - Easy to use and fast - Lower cost *Disadvantages:* - Nebulization of the sample - Element limitations - No screening ability	Wine vinegars, balsamic vinegars	Characterization and distinguishing quick and slow processed vinegars	Guerrero et al., 1997; Del Signore et al., 1998
IRMS, SNIF-NMR	Isotope ratios of bio elements	*Advantages:* - High level of accuracy *Disadvantages:* - Affected by external conditions	Balsamic vinegars	Authentication and detection of adulterations	Camin et al. 2013; Perini et al. 2014
			Wine, apple, malt, cane, beet alcohol vinegars and PDO wine vinegars	Authentication of the origin of vinegars Detection of adulterations	Ortiz-Romero et al. 2018; Raco et al. 2015; Solieri and Giudici 2009

ANEXO III

Chemometrics And Food Traceability

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CHEMOMETRICS AND FOOD TRACEABILITY

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ABSTRACT

Food traceability is currently a priority for the food industries and consumers. Many analytical methods are applied to solve derived challenges for safer food products such as authentication and detection of adulterations. Some challenges have been and are still difficult to solve only by the use of classical analytical approaches. However, the combination of analytical methodologies with chemometrics has demonstrated to be able to address food traceability problems. This chapter provides a review of the most recent applications of analytical methods coupled with chemometrics in food traceability. Some of the most relevant scientific publications are examined in fields like beverages, dairy products, honey, meat and meat products, oils or seafood, among other food products.

KEYWORDS

Food fraud; Food authentication; Food adulteration; Chemometrics; Multivariate data analysis; Chromatography; Spectrometry; Beverages; Oils; Honey; Dairy Products.

NOMENCLATURE

EU: European Union. PDO: Protected Designation of Origin. PGI: Protected Geographical Indication. TSG: Traditional Specialities Guaranteed. HPLC: High Performance Liquid Chromatography. LC: Liquid Chromatography. GC: Gas Chromatography. NIRS: Near Infrared Reflectance Spectroscopy. NMR: Nuclear Magnetic Resonance. ANN: Artificial Neural Networks. DT: Decision Trees. MLR: Multiple Linear Regression. PCA: Principal Component Analysis. PLS: Partial Least Squares. RF: Random Forest. IR: Infrared Reflectance. MIRS: Medium Infrared Reflectance Spectroscopy. MS: Mass Spectrometry. PID: Photo Ionization Detector. FID: Flame Ionization Detector. TCD: Thermal Conductivity Detector. ECD: Electron Capture Detector. MCR: Multiple Curve Resolution. PARAFAC: Parallel Factor Analysis. LDA: Linear Discriminant Analysis. SVM: Support Vector Machine. K-NN: K-Nearest Neighbours. ICA: Independent Component Analysis. TLD: Tri-Linear Decomposition. PCR: Principal Component Regression. QDA: Quadratic Discriminant Analysis. UHT: Ultra High Temperature. FT-IR: Fourier Transform Infrared. ANOVA: Analysis of Variance. SIMCA: Soft Independent Modelling of Class Analogy. GC-FID: Gas Chromatography – Flame Ionisation Detector. GC-MS: Gas Chromatography – Mass Spectrometry. HPLC-DAD: High Performance Liquid Chromatography – Diode Array Detector. HPLC-UV: High Performance Liquid Chromatography – Ultra Violet. ISR: Isotonic Regression. MRI: Magnetic Resonance Imaging. UFGC-MS: Ultra-Fast Gas Chromatography-Mass Spectrometry.

1. FOOD TRACEABILITY

Food traceability is defined as the ability to track and to identify any food at any specified stage of its processing (from production with raw materials to distribution) in order to allow the early detection of any quality problems and safety hazards for efficient recall when needed (Espiñeira and Santaclara, 2016). Food traceability has become increasingly important for the industries and the agro-tech sector since it helps to ensure the quality of the raw products introduced into the food chain, as well as their certifications and accreditations. It is also very important for the consumers, since traceability provides transparency and security about the food products and their nutritional features, reducing the incidences of frauds, adulterations, diseases and environmental emergencies.

The current legislation has developed and implemented specific regulations about traceability that establish control systems throughout the food chain from the raw materials to the available products in the market in order to ensure quality and safety (EU Parlament, 2011; 2016a; 2016b; 2016c). However, several constraints and problems make difficult its implementation. There are two fundamental issues in the traceability systems applied in the food industry for food quality assurance: i) Controlling and authenticating the origin of a product in terms of geographical or botanical/animal provenance and the manufacturing process (food authentication), and ii) detecting adulteration or the addition of a non-declared substance (food frauds).The majority of countries have laws and regulations that require agricultural products to have information about their geographical origin on the labels. The European Union (EU) has encouraged the use of labelling to identify products by introducing regulations in 1992, and more recently in 2016 (EU Parlament, 2016a; 2016b; 2016c). The directives define the following geographical indications for food products: Protected Designation of Origin (PDO), Protected Geographical Indication (PGI) and Traditional Specialities Guaranteed (TSG). The use of these geographical indications implies market recognition, assessment of the quality of the products and, therefore, a fair price in the market. Moreover, it helps to mitigate the food adulteration. Food adulteration is another emerging risk, given the complex, dynamic and global nature of food supply chains. Food adulteration or food fraud is an economically motivated issue that is currently recognized as a great threat to public health (Figure 1).

Different analytical strategies and techniques have been proposed to be useful at different stages of food traceability but mainly in the food process stage. They are mainly based on instrumental techniques such as High Performance Liquid Chromatography (HPLC), Liquid Chromatography (LC), Gas Chromatography (GC), Near Infrared Reflectance Spectrometry (NIRS) or Nuclear Magnetic Resonance (NMR) (Bertacchini et al., 2012; Cajka et al., 2009; Capron et al., 2007; Cocchi et al., 2004; Gonzálvez et al., 2009; Kelly et al., 2005; Mannina et al., 2010; Peres et al., 2007). The use of these techniques entails the collection of a huge volume of analytical data. Unfortunately, not always a big amount of data allows obtaining useful information and knowledge, at least it is not always directly or easily to see. Therefore, algorithms able to extract the desired information from the raw data are strongly needed.

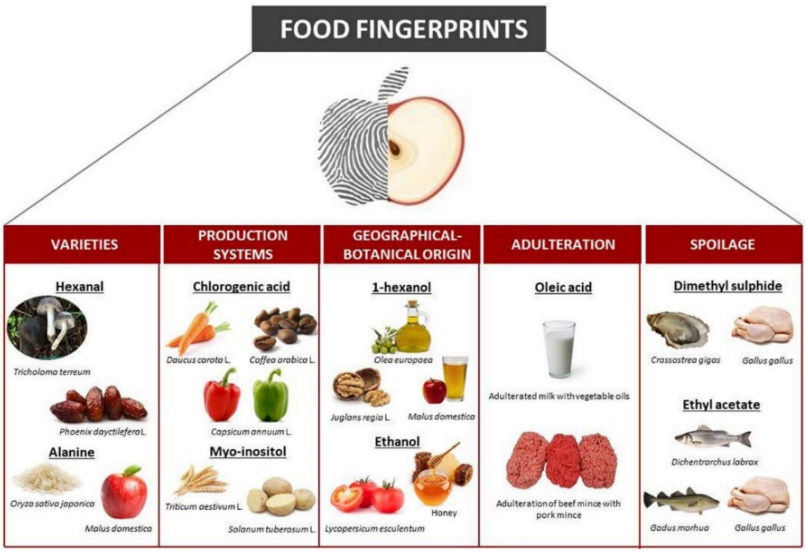

Figure 1. Summary of the main problems that concerns in the food fraud. Extracted from Medina et al., 2019 with permission of Elsevier.

The synergy between the use of analytical techniques and chemometrics represents the most optimal way to obtain feasible results in the development of traceability models (Bertacchini et al., 2013). Thus, the versatility, flexibility, and immediacy of chemometric techniques help in reducing the complexity of data. Chemometrics is a well-known discipline that allows extracting information initially hidden from large datasets in a multivariate way. In this regard, many studies can be found in the scientific literature published in the last years, mainly based in the use of chemometric techniques for the analysis of multivariate signals provided by different instrumental methods (Alamprese et al., 2016; Bosque-Sendra et al., 2012; Camiña et al., 2012; Casale and Simonetti, 2014; Cubero-Leon et al., 2014; Danezis et al., 2016; Domingo et al., 2014; Esslinger et al., 2014; Haddi et al., 2014; Holmes et al., 2012; Kamal and Karoui, 2015; Nascimento et al., 2017). Chemometrics can be seen, as well, as a tool that helps to interact between the analytical information and the final user of the results by "translating" the information obtained by the different analyses and measurements into the same language of the different "speakers" (Scholten et al., 2016).

Thus, the interest in chemometrics has increased because of the decreasing cost of large storage devices and the growing ease in data collection over networks. Other factors that have enhanced the use of chemometrics are the possibility of developing robust and efficient algorithms to process big data, and the increase in computing power, enabling the use of intensive computational methods for data analysis (Fayyad et al., 1996; Mitchell, 1999; Sayad, 2011). However, one of the main drawback of chemometrics is that sometimes it becomes cumbersome to know exactly which multivariate method is the most appropriate for every single type of purpose. In order to solve this problem, many reviews have been published pointing out the main multivariate or statistical methods to be applied for different purposes (Chang et al., 2010; Haaland et al., 2009; Pérez-Palacios et al, 2014; Pierna et al., 2012; Vidal and Amigo, 2012).

This chapter provides a review of the scientific literature related to the application of instrumental techniques and chemometrics on food traceability. Moreover, the main advantages and constraints on this combination in food traceability are also shown and studied in order to define the future challenges on this field.

2. INSTRUMENTAL TECHNIQUES

Nowadays, the strategies applied to detect adulterated or mislabelled products have relied on instrumental techniques mainly because of the increasing level of sophistication of fraud process. Different analytical instrumental techniques have been used to determine the origin of food products as a way to avoid adulterations and mislabelling after the incorporation of food to the market (De La Guardia and Gonzalvez, 2013). Spectroscopy (Abbas et al., 2012; Karoui and Dufour, 2008), GC (Heenan and van Ruth, 2013) and LC (Cserhati et al., 2005) are some of the techniques most used for food authentication. These techniques allow an analysis of the presence of the main components of the sample or some organic compounds that can be characteristics of a specific PDO. However, these organic compounds in food could vary with fertilization, climatic conditions, year of cultivation, history of fields, varieties, geographical location and soil characteristics. This variability affects the authentication of a foodstuff, and therefore, the analytical tools by themselves are sometimes insufficient for this purpose. However, the combination of these techniques with chemometric tools are being studied due to it has shown that it can improve their efficiency and accuracy in this field (Azevedo et al., 2017; Batista et al., 2012; Holmes et al., 2012). These methodologies have demonstrated the possibility to establish whether a product is genuine, to determine the method by which it was made and to ensure that it meets the legal requirements for PDO, that is, in general, they are able to perform traceability testing. Some of these studies are shown in the following sections.

2.1. Spectroscopy

Spectroscopy is widely used for the evaluation of the origin and quality of food. In recent years, many rapid scientific advances in food authentication have taken place due to the fact that an unequivocal decision on the authenticity of a product with conventional methods is not possible in many cases. The association of spectroscopic techniques and chemometrics present a real alternative to the chromatographic fingerprint of foods in order to evaluate the food traceability (Ashurst and Dennis, 1996), due to they are rapid, inexpensive, non-destructive and direct methodologies.

In this sense, Infra-red reflectance (IR) spectroscopy, such as near IR (NIR) or Medium Infra-red reflectance spectroscopy (MIRS) are some of the methodologies that have demonstrated to meet the above mentioned characteristics. NIR is an extremely useful tool for outstanding groups of compounds that have more relevance, giving a fingerprint of each sample, being faster, easier to implement and to use than other techniques. As example, Figure 2 shows the NIR spectral differences between beef and turkey minced meat at different stages (Alamprese et al., 2016).

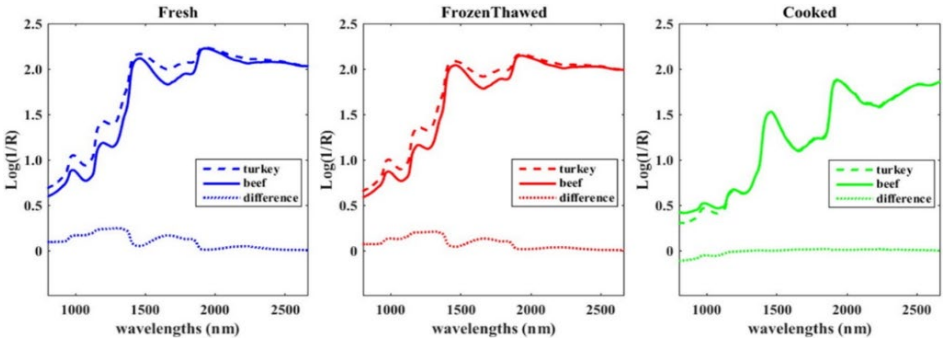

Figure 2. FT-NIR mean spectra of pure beef and turkey minced meat for fresh, frozen-thawed and cooked samples. Differences between beef and turkey spectra are also drawn. Reprinted from (Alamprese et al., 2016) with permission of Elsevier.

However, the main disadvantage of NIRS is that it requires advance multivariate data analysis to allow efficient interpreting of the signals, and therefore, to perform authentication of the samples. The combination of NIRS and chemometrics has demonstrated to be a powerful tool for food classification and authentication according to the origin, the raw material or the production process (Blanco and Pages, 2002; Bevin et al., 2008; Mohamed et al., 2011; Reid et al., 2006; Ríos-Reina et al., 2017a). It was also applied for monitoring the beer production process, even in-line (Grassi et al., 2014), for identifying and detecting adulterations (Lohumi et al., 2015), and even for predicting the concentration or amount of a compound without the need of using a quantitative and expensive analysis (Alamprese et al., 2016; Osborne et al., 1993). Therefore, NIRS can prove real-time measurements of raw or finished food products at all stages of production.

MIRS has also become an important tool for food authenticity and classification purposes (Fernández-González et al., 2014). This method provides information at molecular level and produces a single spectral fingerprint of each sample. It also allows the interpretation of the spectra without the need of complex chemometrics, being this an advantage with respect to NIRS. MIRS was used, in the same way as NIRS, to control certain steps and factors of the production processes in industry, making possible to carry out necessary corrective actions without delay (Durán et al., 2010). Moreover, it has been also used for discriminating PDOs, for differentiating between different raw products, or even for predicting sensory food quality (Guerrero et al., 2010; Pillonel et al., 2003; Ríos-Reina et al., 2017a; Versari et al., 2011), which are important issues in food traceability.

Raman spectroscopy was applied to study water, carbohydrates, proteins and fat structures in food samples and to determine the level of adulteration of virgin olive oil by some vegetable oils such as soybean or corn (Baeten et al., 1996).

NMR is commonly used in combination with stable-isotope measurements. NMR allows the measurement of ^{1}H and ^{13}C and other atoms, having many advantages such as the possibility of obtaining a simultaneous and rapid determination of different metabolites by means of a remarkable reproducibility and repeatability. This makes this technique another useful method for food authenticity and quality control. It has demonstrated to be a useful tool in the analysis of different classes of foodstuffs and beverages for classification and discrimination according to geographical origin, production process and even between organically and conventional food products (Monakhova et al., 2013; Boffo et al., 2009; Consonni et al., 2008; Hohmann et al., 2014).

Moreover, even though fluorescence is one of the oldest analytical methods used, it has also become quite popular as a tool in food technology. Its ability to characterize and classify food samples according to geographical origin, variety, vintage, or the possibility of detecting frauds and monitoring some production process was also demonstrated (Dufour et al., 2006; Dupuy et al., 2005; Karoui et al., 2006; Ríos-Reina et al., 2017b; Sayago et al., 2007)

In spite of these successfully results, it should be taken into account that as spectral data consist of thousands of variables, it can be difficult to interpret without the help of chemometrics (Lohumi et al., 2015). Chemometrics helps to reduce the dimensionality of the data to a smaller number of components concentrating the maximum information. For these reasons, most of the food researches in the literature combine spectroscopic techniques with chemometrics (Table 1).

Table 1. Some applications of spectroscopy and chemometrics techniques for food authentication and food fraud.

Study	Objective	Sample	Chemometrics technique
Bevilacqua et al., 2012	PDO authentication	Olive oil	PLS
Bevin et al., 2008	Classification of different varieties	Wine	PLS, LDA
Bona et al., 2017	Geographical origin	Coffee	PCA, SVM
Chen et al., 2017	Melanime	Milk	PCA
Forina et al., 2015	Geographical origin	Olive oil	PCA, LDA
Godim et al., 2017	Geographical and biological origin	Milk	PCA
Holmes et al., 2012	Greographical origin	Fruits and vegetables	PLS, LDA, RF
Mohamed et al., 2011	Unspecified adulterants	Fruits and vegetables	PCA, ANOVA
Oliveri et al., 2014	Classification of different varieties	Olives in brine	PLS
Ortea and Gallardo, 2015	Geographical and biological origin	shrimps	PCA, KNN
Rios-Reina et al., 2017a	Classification of different categories	Vinegar	PCA
Rios-Reina et al., 2018	PDO authentication	Vinegar	PARAFAC, SVM, PLS
Teye et al., 2015	Classification of different varieties	Cocoa beans	PCA, SVM
Xu et al., 2015	Unspecified adulterants	Kudzu starch	PLS
Zhao et al., 2014	Geographical origin	Beef	PLS, LDA

2.2. Chromatography

Among the separation techniques for food characterization, LC was extensively used in food analysis for measuring numerous organic compounds, such as, carbohydrates, vitamins, additives, mycotoxins, amino acids, and proteins. LC is a robust, relatively low cost and reproducible technique, which can be coupled to different detector depending of the studied analysis. This technique has been widely used in food analysis and authentication (Guijarro-Diez et al., 2015; Jandríc et al., 2015; Jandric et al., 2014; 2017; Popping et al., 2017; Prandi et al., 2017; Vaclacik et al., 2011; Vuckovic, 2012; Wulff et al., 2013).

On the other hand, GC is one the most universal separation techniques used in food analysis, mainly for volatile and semi volatile compounds studies, and for other type of studies such as aroma or pesticides (Hajslova and Lehotay, 2002). In the same way, different detectors were applied depending on the analysis types: Mass Spectrometry (MS), Photo-Ionization Detector (PID), Flame-Ionization Detector (FID), Thermal Conductivity Detector (TCD) and Electron Capture Detector (ECD). This technique was widely used in food quality control and authentication (Black et al., 2016; Cavanna et al., 2018; Gil-Solsona et al., 2016; Huck et al., 2016; Kus and Van Ruth, 2015; Novotna et al., 2012; Ozdestan et al., 2013; Silva et al., 2017). However, regardless of the fact that this technique has been widely employed in food, its experimental sources of variability (e.g. columns, stationary phase, experimental conditions and sample preparation) still cause some variations that directly affects the results. These problems were resolved by chemometric tools such as multiple curve resolution (MCR) or Parallel factor analysis (PARAFAC) (Amigo et al. 2008; Hantao et al., 2012). Table 2 shows more examples about food authentication and traceability by using GC and LC.

Table 2. Some applications of chromatography and chemometrics techniques for food traceability.

Study	Objective	Sample	Chemometric technique	Chromatography technique
Aranda et al., 2004	Classification of the commercial virgin olive oil samples	Oil	PCA, LDA	LC
Azevedo et al., 2017	Geographical origin	Honey	PCA	GC
Chiesa et al., 2016	PDO	Lard	LDA	GC
Cuevas et al., 2017	Assessment of premium organic oranges juices authenticity	Orange Juice	LDA	GC
Feudo et al., 2011	Geographical origin of tomatoes	Tomato	LDA	GC
Jabeur et al., 2016	Distinction and detection of adulteration on cheap olive oils	Olive oil	LDA	GC
Jablonski et al., 2014	Detection of adulteration of skim milk powder with foreign proteins	Milk	SIMCA	LC
Jimenez-Carvelo et al., 2017	Types of vegetal oil	Oil	PCA, SVM	LC
Kalagouri et al., 2016	Quantification of quality of the samples	Oil	PLS, LDA	LC
Kim et al., 2014	Discrimination of commercial cheese	Cheese	PCA	GC
Maia and Nunes, 2013	Geographical origin	Honey	PCA	GC
Malheiro et al., 2013	Botanical species	Mushrooms	PCA	GC
Nayik et al., 2018	Discrimination of high altitude Indian honey	Honey	PCA, LDA	LC
Nescatelli et al., 2014	Discrimination of the PDO of olives oil	Oil	PLS, LDA	LC
Obiesan et al., 2017	Determination of origin varieties	Oil	PCA, LDA	LC
Pauli et al., 2014	Detection of ground roasted coffee adulteration with roasted soybean and wheat	Coffee	PCA, LDA	LC
Reid et al., 2004	Differentiation of apple juices samples	Apple Juice	PLS	GC
Rodríguez et al., 2010	Detection of adulteration of ovine, caprine and bovine milk	Milk	PCA, PLS	LC
Rodríguez-Bermudez et al., 2018	Authentication of the organic status	Milk	MLR	GC
Ruiz-Samblas et al., 2011	Classification of olive oil varieties	Olive oil	PCA	GC
Sarbu et al., 2012	Classification according to the fruit species and subspecies	Kiwi and Pomelo	PCA	LC
Serrano-Lourido et al., 2012	Geographical origin	Wines	PCA, PLS, LDA	LC
Silvestri et al., 2014	Classification of different varieties	Wines	PCA, PLS, LDA	LC
Stanimirova et al., 2010	Geographical origin	Honey	LDA	GC
Sun et al., 2015	Adulteration detection	Flexseed oil	SVM	GC
Surowiec et al., 2011	Detection of mechanically recovered meat	Meat	PCA	GC
Tavares et al., 2016	Detection of Arabica coffee adulteration with maize	Coffee	PCA, LDA	LC
Toledo et al., 2014	Detection and quantification of coffee adulteration	Coffee	PLS	GC
Versari et al., 2008	Characterization of Italian commercial apricot juices	Apricot juices	PCA	LC
Zhang et al., 2017	Geographical origin	Sea Cucumber	PCA	GC

3. CHEMOMETRICS

Chemometrics is an analytical discipline that uses statistical, mathematical and data analysis methods to achieve objective data evaluation by extracting the most important information from related and unrelated collections of chemical data by using mathematical and statistic tools (Massart et al., 1997). Chemometrics (a.k.a. multivariate data analysis) has demonstrated to have many applications in quantitative and qualitative determination of chemical parameters for assessing the food-products authenticity (Yu et al., 2018). It provides powerful results in targeted and non-targeted approaches to identify various food fraud situations or to certificate their geographic or biological origin (Beale et al., 2017; Martínez-Bueno et al., 2018). Chemometrics methods can be grouped in many different ways. The most straightforward one is attending to their main purpose: Pattern recognition, resolution, regression and classification. Figure 2 summarizes the most popular methods within each group, acknowledging that there are many similar methods to the ones cited in this figure.

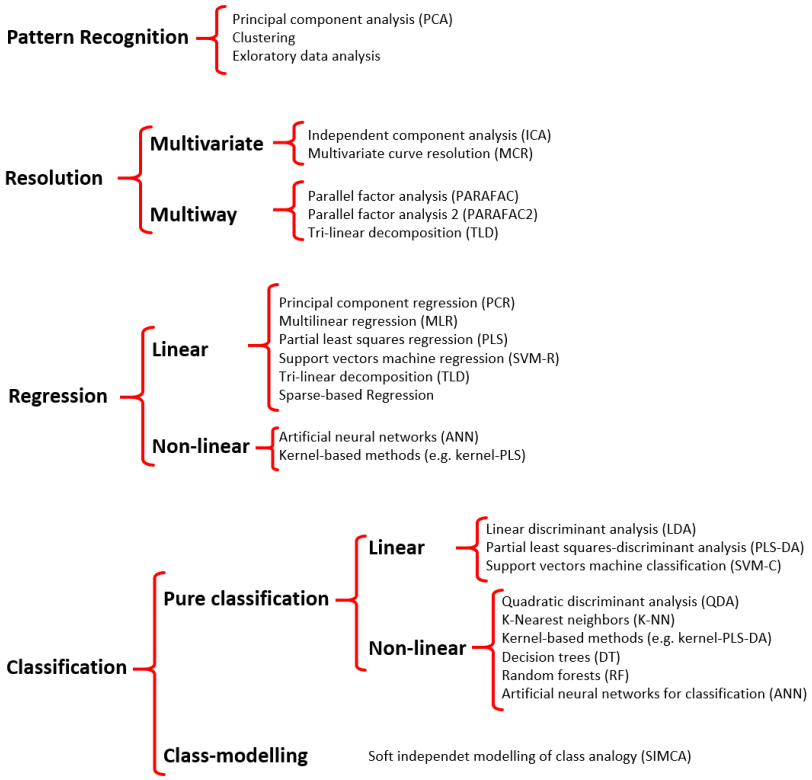

Figure 3. Scheme of the main chemometric methods applied in the context of food fraud detection and traceability

Pattern recognition

Pattern recognition are, among the four groups denoted in Figure 3, the only ones that can be purely denoted as not supervised. That is, they do not need a previous step of calibration in order to find hidden patterns in the data. The purpose of the unsupervised methods is to identify clusters or relationships between samples, without any prior knowledge of classes or groups. They are used to study if an unknown sample is similar or not to set of authentic ones. Among the unsupervised methods, the most common method used in any kind of data is principal component analysis (PCA). PCA is useful in order to elucidate the complex nature of multivariate relationships by using mapping and displaying techniques for understanding the structure of complex multivariate data sets (Bro et al., 2002; Elmqvist and Fekete, 2010).

Curve resolution methods

Curve resolution methods aim at resolving mixtures, given the correct number of constituents, their response profiles (e.g. spectral, time or elution profiles) and their relative signal influence on the sample (Amigo et al., 2010a). It is not the point herein to discuss about the supervised or unsupervised nature of curve resolution methods. Here we will only highlight that in most of the situation, curve resolution methods need some a-priory information (number of components, sensible initial estimations, selectivity information) in order to minimize the big issue of the ambiguities that these methodologies have. Despite this fact, curve resolution methods in their both version (Multivariate or multiway) have been widely used mostly in chromatography, where they have demonstrated their versatility of solving common chromatographic issues and resolving peaks that are difficult to resolve otherwise. An example of this is displayed in Figure 4, where the versatility of parallel factor analysis 2 (PARAFAC2) is shown in solving different common chromatographic issues in the monitoring of the evolution of apples being ripened.

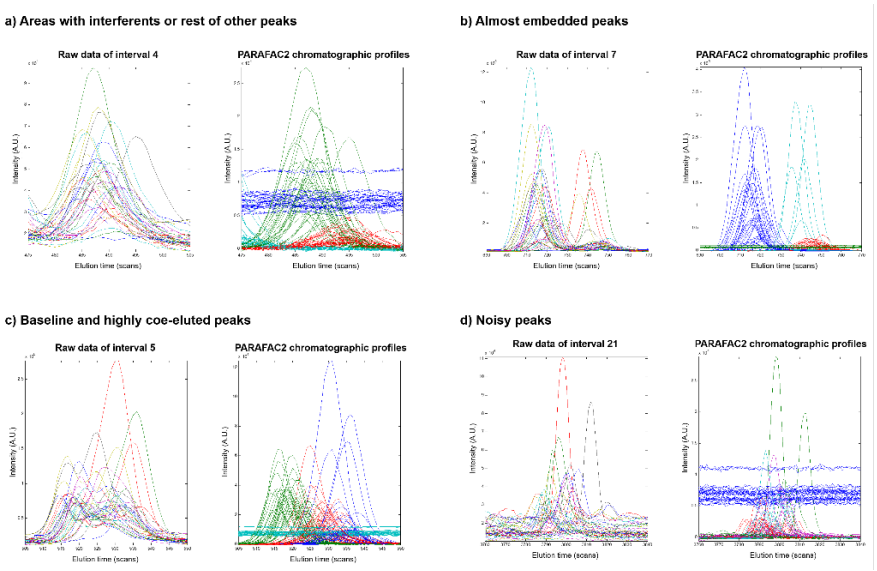

Figure 4. PARAFAC2 results obtained in several intervals denoting different chromatographic problems. In all the cases, the left plot corresponds to the raw data; whereas the right plot corresponds to the resolved chromatographic profiles obtained by PARAFAC2. Figure extracted from (Amigo et al., 2010b) with permission of Elsevier.

Regression and classification in a multivariate context. Aspects to consider

Calculating the concentration of several compounds in foodstuff (regression) and, specially, the classification of samples in different well-defined categories (classification) is one of the major targets in food-related issues. Normally, they are also known as supervised methods, since a set of samples were the property to measure or the belonging to a specific class must be well known. Therefore, a calibration (training) step is performed and, afterwards, unknown properties are predicted afterwards (Caballero et al., 2018c; Witten and Frank, 2005; Wu et al., 2008). In this concern, we can find the main algorithms for linear or non-linear training models like PLS, MLR, SVM or ANN, among others, being adapted for regression or classification issues; or algorithms specifically designed for an specific target, as linear discriminant analysis (LDA) or SIMCA (Esteki et al., 2018; González-Fernández et al., 2018; Granato et al., 2018; Lendhart et al., 2015; Ma et al., 2016; Sampaio et al., 2018; Yudthavorait et al., 2014).

The quality and the accuracy of regression and classification methods depend on many factors to be taken into account from the very beginning of the analysis (i.e. from the sampling

stage). The representativeness of the samples, the quality of the data reached in the analysis, the presence of outliers and the mandatory validation step are aspects to thoughtfully consider. Moreover, they are connected within them. That is, if the proper amount of representative samples is used, the validation procedure will lead to a more reliable model. Nevertheless, in many cases, collecting an appropriate number of samples that span the plausible variability of natural food products might be cumbersome. Different varieties, families, cultivar conditions, the inner variability of the product itself, producers, composition and manufacturing, year of production, location, pesticides treatments or flavour powder are some of the many factors that can make that your models do not collect all the desirable variability. Thus, each sample must be described as much as possible to give an account of any kind of source of variability (Forina et al., 1991; Maninna et al., 2010; Millan et al., 1998; Vandeginste, 2013). This point is extremely important in validation.

Needless to say, all supervised method (either for regression/calibration or classification) must be properly validated. Validation is the essential step that gives a real account of the real capability of our model to calculate a concentration or predict a class. Many types of validations can be applied. It is not the point here to describe all of them. Nevertheless, it is extremely important to remark that the whole variability of the samples must be represented in both calibration and validation sets.

Another important point in natural products is the possibility of having missing values and different types of outliers. Their presence reduces the data available to be analysed compromising the statistical power of the study and the reliability of the results (Kwak and Kim, 2017). As part of the pre-treatment process of data, missing values need to be replaced. Among the different methods available for treating and analysing missing values (Kwak and Kim, 2017), the two most extended procedures are the principal component imputation and the application of infer techniques of data mining (Pérez-Palacios et al., 2014; Stanimirova and Walczak, 2008). After properly building and organizing the data matrix, it is also necessary to perform data preprocessing—that is also another crucial step in multivariate analysis (Bro, 1998). Preprocessing is required to make the distributions of the different variables symmetrical in order to eliminate or reduce sources of variations due to analytical responses and to obtain more efficient data from which meaningful information can be extracted. There are many different preprocessing methodologies, and the selection of the suitable preprocessing procedure mainly depends on the nature of the data (Rinnan et al., 2009).

4. APPLICATIONS

Traceability is a vital issue for food supply chains, industries and consumers to guarantee food quality and provenance. Nowadays, due to the increased globalization of food markets, many food products are supplied from different countries. Therefore, detection and trace the source of intentional contamination would be difficult especially in highly processed foods. In general, foods and food ingredients that usually suffer fraud problems are wine, fruit juices and beverages, milk and dairy products, honey, meat and meat products, seafood, and other organic and processed foods (Bertacchini et al., 2013; Bianchi et al., 2018; Cajka et al., 2010; Callao and Ruisánchez, 2018; Cavanna et al., 2018; Esteki et al., 2018; Leardi et al., 2018; Versari et al., 2014). In the following sections, some examples show the potential of the aforementioned analytical and statistical tools in the performance of traceability, authentication, and detection of frauds.

4.1. Beverages

Fluid foods, such as wines, tea, coffee or fruit juices and other beverages are considered the major products of the global fruit-processing industry. However, they are also one of the products more susceptible to be adulterated, since they can be easily mixed with a number of cheaper liquids. This action not only produce low quality products, but also might create toxic compounds that may lead to serious health risks. Fruit juice is a major target for adulteration by its simple dilution with water or by the mixture with juices obtained from cheaper fruits, as well as by an undeclared addition of sugar syrups, pulp wash, acids and colorant agents (Ogrinc et al., 2003). Other regulatory concern is also ascertaining the geographical origin of a juice. While numerous analytical methods were studied to detect juice adulteration, only some of them have proven workable in deterring these adulterations. Thus, for example, an untargeted metabolomics approach by using LC coupled with PLS-DA was able to authenticate berry fruit juice and to discriminate it from their adulterant apple and grape juices (Zhang et al., 2018). There are other successful examples about juice authentication and detection of adulteration by spectroscopic data and chemometric analyses (see Table 3).

Table 3. Applications on beverages for food authentication and food frauds.

Study	Objective	Sample	Chemometric technique	Analytical technique
Coelho et al., 2018	Simultaneous analysis of sugars and organic acids in wine	Wine	PCA	HPLC
Cuevas et al., 2017	Assessment of premium organic orange juices authenticity	Orange juices	PLS, LDA	GC-MS
Dall'asta et al., 2011	Differentiation of wine brands	Wines	PCA	GC-MS
De Luca et al., 2016	Characterization of the effects of different roasting conditions on coffee samples of different geographical origins	Coffee	PLS, LDA, SIMCA	HPLC-DAD
Domingues et al., 2014	Detection of roasted and ground coffee adulteration	Coffee	PCA	HPLC
Fraser et al., 2013	Geographical origin of tea	Tea	PCA, PLS, LDA	HPLC
Guo et al., 2012	Discrimination of juices by variety and geographical origin	Apple juices	PCA, LDA	GC-MS
Jiao et al., 2011	Classification by types, breweries and tastes	Beer	PCA	GC-FID
Larrauri et al., 2017	Determination of polyphenols in white wines	Wines	PCA	HPLC-UV
Nogueira and Do Lago, 2016	Adulteration with different cereals	Coffee	PCA	GC-MS
Parastar et al., 2012	Classification by fruit types	Citrus fruits juices	MLR	GC-MS
Pongsuwan et al., 2008	Prediction of product quality	Tea	PLS	GC-MS
Reid et al., 2004	Differentiation of apple juice samples	Apple Juices	PCA, PLS	GC-MS
Ribeiro et al., 2012	Modelling of sensory prediction	Coffee	PLS	GC-FID
Salvatore et al., 2013	Authentication of geographical origin	Wines	PLS, LDA, SIMCA	GC-MS
Serrano-Lourido et al., 2012	Determination of geographical origin	Wines	PCA, PLS, LDA	HPLC
Socha et al., 2015	Characterization of Polish wines	Wines	PCA	HPLC
Thimmaraju and Yao, 2015	Differentiation of geographic origin of coffee beans	Coffee	PCA	GC-MS
Toledo et al., 2014	Detection and quantification of coffee adulteration	Coffee	PLS	GC-MS
Tredoux et al., 2008	Varietal origin of wines and/or grapes	Wines	PCA, LDA	GC-MS
Ye et al., 2012	Geographical origin of tea	Tea	PCA, LDA	GC-MS
Zhao et al., 2011	Differentiation between tea categories	Tea	PCA	HPLC-DAD

The value of wine and its wide market made the wine authentication and traceability important tasks worldwide (Cozzolino et al., 2009). Variety, provenance, year of production, process of vinification and quality ratings, that are the most important parameters used to specify, can be affected by the composition of small molecules (Cuadros-Inostroza et al., 2010). In the past decades, the only adopted method for characterization and discrimination of wines was based on sensory evaluation performed by a panel of experts (Ballester et al., 2008). It is obvious that the modern wine industry needs both fast and reliable quality control methods that allow fast and efficient analysis to assess the quality of the final product to the consumer.

Moreover, many wines, and its derivate vinegars, are recognised under a PDO, which provides them a higher added value for the market and consumers, but also makes them more vulnerable to frequent frauds. Adulteration of wine or other alcoholic beverages is usually accomplished by dilution with water, addition of alcohol, dyes and aromas, and mixing with lower quality grapes (Stanziani, 2009). Beside these types of adulteration, mislabelling about composition, variety of the grapes or geographical origin could be carried out with wine, vinegar and other beverages (Ohtsubo et al., 2008). By searching in the literature, many analytical techniques were applied for the authentication of these products, the classification of PDOs, the detection of frauds and traceability testing, being the most recently one the spectroscopic and metabolomics methods combined with chemometrics (see Table 3), (Arvanitoyannis et al., 1999; Tesfaye et al., 2002).

Tea is one of the most popular beverages in a wide range of countries. In general, green and oolong tea are consumed mostly in Asian countries such as India, China, Japan and Thailand, while black tea is more popular in Western countries (Sharangi, 2009). Adulteration of tea usually involves addition of colorants or mixing with tea from other geographical origins or lower quality grades. In this way, appropriate analytical methods are important tools for monitoring adulteration activities and preventing incorrect labelling. Another of the most consumed beverages is coffee (Roberts, 2016). However, in this case, the adulteration of coffee is usually performed to reduce costs (Toci et al., 2016). Moreover, it is also applied by addition of substances as coffee husks, corn and barley, cereals or caramelized sugar, in order to reach less expensive product (Nogueira and Do Lago, 2009; Pauli et al., 2014), or even mixing different varieties of coffee beans (Calvini et al., 2015; 2017).

4.2. Dairy products

Milk and dairy products with highly nutritive value make a great contribution to a healthy diet for different groups of consumers (Jenkins and McGuire, 2006). Milk as one of the top-adulterated food products could be counterfeited by several ways (Borková and Snášelová, 2005). Mixing with different types of milk (Dias et al., 2009) and addition of melamine (Jawaid et al., 2013), salt or sugar (Nirwal et al., 2013) to mask extra water or high solid contents are some instances. Commercial ultra-high temperature milks (UHT) can be presented by addition of adulterants such as starch, chlorine, etc. (Souza et al., 2011). Excessive addition of water into milk leading the decrease of nutritional composition (Mabrook and Petty, 2003), and addition of non-milk fat into dairy products (Kamal and Karoui, 2015; Nascimento et al., 2017) are other counterfeiting methods which generally used (Table 4).

Table 4. Applications on milk dairy products for food authentication and food frauds.

Study	Objective	Sample	Chemometric technique	Analytical technique
Cossignani et al., 2011	Detection of cow milk in donkey milk	Milk	LDA	GC-FID
Domingo et al., 2014	Geographical origin	Milk	PLS-DA, SVM	HPLC
Gan et al., 2016	Classification and prediction of Cheddar cheese maturity	Cheese	PLS-DA, LDA	GC-MS
Jablonski et al., 2014	Detection of adulteration of milk powder with foreign proteins	Milk powder	SIMCA	HPLC
Kim et al., 2014	Detection for non-milk fat in dairy product	Cheese	PCA	GC-FID
Majcher et al., 2015	Authentication of geographical origin	Cheese	PCA, LDA, SVM	GC-MS
Rodríguez et al., 2010	Detection of adulteration of ovine, caprine and bovine milk	Milk	PCA	HPLC-DAD
Santos et al., 2016	Detection of adulteration of milk	Milk	PCA, SIMCA, KNN	NMR
Tociu et al., 2017	Authentication Cheeses	Cheese	PCA	GC-FID
Trbovic et al., 2017	Detection of adulteration of milk fat	Milk fat	MLR, PCA	GC-MS
Wu et al., 2016	Identification and quantification of adulterants in milk	Milk	PLS-DA, SIMCA	NIRs

The authenticity of dairy products could also be attributed to their geographical origin (Osorio et al., 2015) and processing technology (Schmidt and Mayer, 2018). Labelling of conventional milk as a product from organic farming is another issue related to the authenticity of milk and dairy products and this should be considered (Erich et al., 2015; Sardina et al., 2015; Di Domenico et al., 2017). Traceability of dairy products is also an important issue indicating the presence of undesirable compounds in order to protect consumers from harmful contamination (Motarjemi et al., 2014). Although authenticity issues of dairy products mostly include detecting of the adulteration, traceability and safety, other factors such as processing conditions and packaging could also be considered.

The dairy industries and milk recording agencies are already using spectroscopic techniques coupled with chemometrics as solutions to some extent. For example, high-throughput Fourier Transform Infrared (FT-IR) based technologies are routinely applied in the dairy industry during milk standardization (fat, protein and lactose) and as routinely milk analyses. Moreover, the detection of contaminants such as Melamine or fat adulterants (Domingo et al., 2014; Trbović et al., 2017; Wu et al., 2016), the discrimination of the geographical origins of milks (Domingo et al., 2014), and organic milk authentication (Liu et al., 2018; Molkentin, 2009; Rodríguez-Bermúdez et al., 2018), are other studies carried out with different analytical methods and chemometrics (Table 4).

4.3. Honey

Honey is a rich conventional natural resource with a specific physico-chemical traits. Their quality traits are mainly determined by their sensorial, chemical, physical and microbiological characteristics that in turn depend on their chemical composition, floral origin, production method, thermal treatment, climatic conditions of the region and the conditions of manipulating and packaging (Alvarez-Suarez et al., 2010; Turhan et al., 2008).

Honey has the potential to be used in a variety of applications in the food industry. It can be used by direct consumption or incorporated as an ingredient in various processed food products

for their characteristics (Azeredo et al., 2003; Kahraman et al., 2010). Because of its nutritional value and unique flavour, and considering its production cost, the price of natural honey is much higher than that of other sweeteners. Therefore, honey is another main target of food adulteration. Changing the composition of honey by adding other sweet ingredients in any part of the production or processing can be an attractive way to achieve financial benefits (Table 5).

Adulteration of honey by adding cheap sweet ingredients was reported in the literature (Downey et al., 2003; Kelly et al., 2004; Li et al., 2012). It is usually difficult to detect this kind of adulteration, because the sugar compounds of these cheap syrups are sometimes close to honey. Moreover, adulteration of honey can be produced by feeding bees with artificial sources (Kast and Roetschi, 2017) and incorrect information about the honey's geographic and botanical origin lead to serious problems for honey producers and consumers (Bougrini et al., 2016).

Table 5. Applications on honey for food authentication and food frauds.

Study	Objective	Sample	Chemometric technique	Analytical technique
Azevedo et al., 2017	Authentication of geographical origin	Honey	PCA	GC-MS
Chen et al., 2017	Classification of the botanical origin of Chinese honey	Honey	PCA	GC-MS
Gerhardt et al., 2018	Assessment of the authenticity of honey	Honey	PCA, LDA, KNN	GC-MS
Kus and Van Ruth, 2015	Discrimination of Polish unifloral honeys	Honey	PCA	HPLC-DAD
Nozal et al., 2005	Geographical origin	Honey	LDA	HPLC-DAD
Revell et al., 2014	Classification of honey based on floral source	Honey	PCA	GC-MS
Stanimirova et al., 2010	Geographical origin	Honey	LDA, PLS, SVM	GC-MS
Sun et al., 2017	Identification of botanical origin of Chinese unifloral honeys	Honey	PCA, LDA	HPLC

4.4. Meat and meat products

In the last years, the interest on meat authenticity and traceability has increased. The need to specify the information about origin, accurate labelling, ingredients and allergens of meat products is growing rapidly due to increased awareness of consumers about their health risks. Five main categories are considered in traceability of meat and meat products: meat species, production processing, production treatment, geographic origin and non-meat ingredient addition (Ballin, 2010). In addition, the need of unambiguous and accurate labelling of meat and meat products is important to reassure consumers, protect regional designations and ensure fair competition. There is a strong preference and willingness to pay a higher price for particularly PGI and PDO meat products which almost are organic products from selected breeds produced in a particular area (Deselnicu et al., 2013; Pla et al., 2007). In this context, many spectroscopic techniques in conjunction with chemometrics, such as multivariate regression and class-modelling strategies, were applied for detecting adulterations, discriminating between different productions and authenticating meat (Table 6).

Table 6. Applications on meat and meat products for food authentication and food frauds.

Study	Objective	Sample	Chemometric technique	Analytical technique
Alamprese et al., 2016	Adulteration in fresh, frozen and thawed meat	Turkey meat	PCA, PLS, LDA	NIRS
Caballero et al., 2017	Prediction of physico-chemical of pork meat	Pork meat	MLR, ISR	MRI
Caballero et al., 2018a	Prediction of sensory traits of pork meat	Pork meat	MLR, ISR	MRI
Caballero et al., 2018b	Discriminating genetic lines of Iberian pigs	Pork meat	LDA	GC-MS
Gorska-Horczyczak et al., 2017	Quality control of the pork meat	Pork meat	PCA, ANN	UFGC-MS
Olaoye et al., 2018	Assessment of the effect of different packaging materials on some quality indices of a Nigerian stick meat during Storage	Stick meat	PCA	HPLC
Zhao et al., 2014	Geographical origin	Beef meat	SIMCA, PLS, LDA	MIRS

4.5. Oils

Nowadays, vegetable oils, salad and cooking oils, margarine and butter, are classified as the foods which are most frequently susceptible to adulteration (Hong et al., 2017). Adulteration in oils has become one of the most important food safety issues because it has seriously affected peoples' health (Picouet et al., 2018). Thus, the replacement of expensive oils with cheaper fats and the mixing cold press oil with a refined one are two major ways of adulteration of oils (Azadmard-Damirchi and Torbati, 2015).

Olive oil, especially with regard to its high demand and profit potential, is a significant target for economically motivated adulteration. The chemical composition of the olive oils differs due to the fruit cultivar, degree of fruit ripeness, environmental and storage conditions, geographical production area and the extraction methods (Kalua et al., 2007). These properties not only affect the fatty acid composition of the oil, but also especially regulate the levels of diverse classes of minor compounds.

Geographical origin of olive oil production is another important choice factor for consumers (Benincasa et al., 2007; Menapace et al., 2011). Identification of the geographical origin of olive oil is an indispensable issue to ensure the high quality of the extra virgin olive oils that shows the specific quality traits attributed to their geographical origin (Bendini et al., 2007). According to the aforementioned issues in oil traceability, a huge number of researches have been focus on the authentication, differentiation, characterization, quality control and detection of frauds and contaminants in oils. These studies applied spectroscopic techniques such as fluorescence and visible and infrared spectroscopy (Dupuy et al., 2005; Guimet et al., 2005; Öztürk et al., 2010; Pizarro et al., 2013; Sayago et al., 2007; Tena et al., 2009). NMR (Alonso-salces et al.,2012; Camin et al., 2010; Mannina and Sobolev, 2012; Portarena et al., 2015; 2017), as well as chromatographic techniques (Romero et al.,2015; Yagüe et al., 2005), being the majority of them supported by their combination with multivariate data analysis such as PCA, PLS-DA, PLS regression, LDA or three-way chemometric methods (Table 7).

Table 7. Applications on oils for food authentication and food frauds.

Study	Objective	Sample	Chemometric technique	Analytical technique
Aranda et al., 2004	classification of the commercial virgin olive oil samples	Olive oil	PCA, LDA	HPLC
Bevilacqua et al., 2012	Determination of PDO	Olive oil	PLS	NIRS
Forina et al., 2015	Authentication of geographical origin	Olive oil	PCA, LDA	NIRS
Jabeur et al., 2016	Distinction and detection of adulteration on cheap olive oils	Olive oil	LDA	GC-FID
Jimenez-Carvelo et al., 2017	Types of vegetal oil	Vegetal Oil	PCA, SVM	HPLC
Kalogiouri et al., 2016	Quantification of quality of the samples	Olive oil	PLS, LDA	HPLC
Mansor et al., 2011	Detection of lard adulteration in virgin coconut oil	Coconut oil	LDA	GC-MS
Melucci et al., 2016	Discrimination of geographical origin of extra virgin olive oils	Olive oil	PCA, PLS, LDA	GC-MS
Nescatelli et al., 2014	Discrimination of the PDO of olives oil	Olive Oil	PLS, LDA	HPLC
Obiesan et al., 2017	Determination of origin varieties	Olive Oil	PCA, LDA	HPLC
Ruiz-Samblas et al., 2011	Classification of olives oil varieties	Olive oil	PCA	GC-MS
Sun et al., 2015	Adulteration detection	Flexseed oil	SVM	GC-MS

4.6. Other food products

Fish and seafood fraud usually involves a deliberate increase in product weight and the use of prohibited additives in production. The addition of excess water to frozen product, or the partial substitution by cheaper seafood are some examples of problems in fish traceability (Moore et al., 2012). Moreover, many consumers are increasingly aware of nutritional and environmental issues regarding fisheries, size of species, catch location, and methods of fishing (Armenta and de la Guardia, 2016). Several methods such as stable isotope analysis have demonstrated to be capable of identifying the authenticity and traceability of seafood by means of identifying the production method (wild or farmed), the geographical origin, and biological species. However, as these methods are either time-consuming or have limitations for practical uses, new analytical methods to authenticate these products in terms of rearing systems used, species identification, geographical origin and processing and storing methods are being studied (Table 8).

Regarding eggs, similar researches have been developed by using traditional analytical techniques such as stable isotope analysis, which can provide valuable information about the feeding regimen administered to hens and their housing system (Capuano et al., 2013), and also by applying new analytical and chemometric tools such as fluorescence spectroscopy or NIRS coupled with SVM to determining the egg freshness (Sádecká and Tóthová, 2007; Zhao et al., 2010).

Other products with the need of authentication and traceability control due to their high value and quality are fruits and vegetables. There are numerous works in the literature with the aim of identifying the geographical origin or PDO of some of these products, as tomatoes, onions, potatoes or cherries (Armenta and de la Guardia, 2016; Cubero-Leon et al., 2014). Moreover, cultivars discrimination and quality determination are other vegetable and fruit

traceability problems also taken into account in the industry and scientific researches (Cubero-Leon et al., 2014; Mohamed et al., 2011). For this traceability testing, the combination of chemometrics and analytical techniques has provided satisfactory results.

Table 8. Applications on other food products for food traceability.

Study	Objective	Sample	Chemometric technique	Analytical technique
Chen et al., 2009	Identification of Raspberries	Raspberry	PCA	HPLC-DAD
Durante et al., 2006	Prediction of sensory attributes	Vinegars	PLS	GC-FID
Feudo et al., 2011	Geographical origin of tomatoes	Tomato	LDA	GC-MS
Malheiro et al., 2013	Botanical species	Mushrooms	PCA	GC-MS
Oliveri et al., 2014	Classification of different varieties	Olives in brine	PLS	NIRS
Ortea and Gallardo, 2015	Geographical and biological origin	shrimps	PCA, KNN	NIRS
Rios-Reina et al., 2017a	Classification of different categories	Vinegar	PCA	FT-IR
Rios-Reina et al., 2017b	PDO authentication	Vinegar	PARAFAC, SVM, PLS	Fluorescence
Rios-Reina et al., 2018	PDO authentication	Vinegar	PCA, PLS-DA	NIRs
Sarbu et al., 2012	Classification according to the fruit species and subspecies	Kiwi and Pomelo	PCA	HPLC
Teye et al., 2015	Classification of different varieties	Cocoa beans	PCA, SVM	NIRS
Zhang et al., 2017	Geographical origin	Sea Cucumber	PCA	GC-MS

5. FUTURE TRENDS AND PERSPECTIVES

Over the past decade, due to recent crises and food industry scandals that have affected the confidence of the consumers, the concern for quality of food products and production methods has increased significantly. For this reason, it is important to ensure the quality of the raw products introduced into the food chain, and the certifications and accreditations of their products, since it provides transparency and security about the products and their nutritional parameters.

The combination of powerful analytical platforms and chemometrics have addressed and are addressing the above mentioned issues in a brilliant attempt to mark guides for food traceability monitoring and fraud detection. Regulatory agencies and quality laboratories are adopting and adapting this binomial synergy and soon more official protocols are expected to be found.

Moreover, the advantage of having the availability of more sophisticated instruments in normal research laboratories or the combined action of several laboratories have raised a new trend for applying data fusion methodologies. Profiting the synergy between different spectroscopic methods and proper data analysis can lead to a knowledge of a sample that promotes the generation of a singular fingerprint of that sample (Figure 5) and thus making more difficult the possibilities of adulteration or counterfeits.

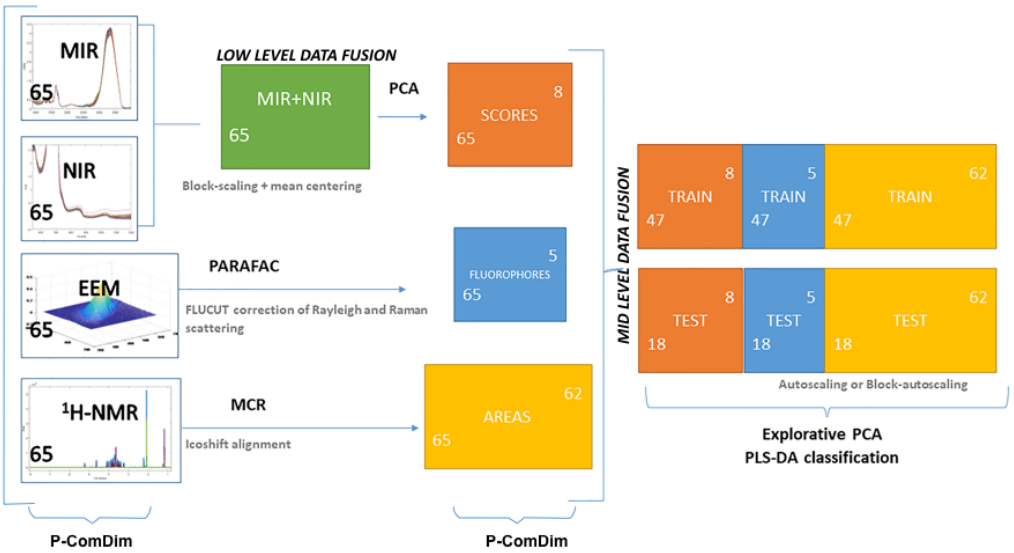

Figure 5. Graphical representation of the data sets, data analysis flow and data fusion process. Reproduced from (Ríos-Reina et al. 2019) with permission of Elsevier.

Combining information from different instrumental sources can improve the results, of course. Nevertheless, depending on the problem and on the maximum permitted error, the improvement has to be carefully evaluated in term of cost-benefit ratio. Thus, although spectroscopic measurements (the most used techniques) are rapid and economical, measuring a food product by more than one of them could represent an additional cost.

Last but not least, and coming back to the validation problem, a bigger effort should be made to create robust and reliable analytical databases of the raw food or processed food in order to generate quality regression or classification models. Depending on the problem, this is one of the key points that must be thoughtfully consider.

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